ASPECTS OF THE NORMAL HUMAN BREAST

SARAH LENNOX MANTON

Thesis presented for the degree of
Doctor of Philosophy
of the University of Edinburgh
1983

DECLARATION

With the exception of acknowledged work, the composition of this Thesis, and the investigative procedures described were designed and performed by the author.

SARAH L. MANTON
May, 1983.

Abstract

Studies have been made on normal tissue from 50 whole breasts and 43 breast biopsies using subgross histological and duct injection techniques. The results obtained from the various investigations have been compared with factors which may affect the breast, notably age, the menstrual cycle, parity and laterality.

It has been found that the ducts in the breast do not branch in a simple dichotomous manner, nor are individual lactiferous systems arranged about the nipple in discrete lobes like the spokes of a wheel. Rather, the duct systems are interwoven with each other in an intricate manner and may not be dissected out individually.

The quantity of parenchymatous lobules in the breast was found to decrease significantly with age. Lobules occurred in areas of high focal density and their number was not related to the amount of fibrous stroma present. Lobules did not occur with greater frequency in the upper outer quadrant of the breast and lobule number was not related to tumour incidence. Parity and laterality were without significance in the present study.

The presence of acid mucopolysaccharides in the intra-lobular breast stroma showed a cyclical variation which peaked at approximately day 24 of the menstrual cycle. It is suggested that hyaluronic acid is the principal component of the staining reaction and may be associated with premenstrual water retention in the breast. The incidence of intraluminal secretions within the ductules of lobules, and intracellular granules within ductular

cells, did not reveal cyclical variation.

Normal lobule types occurring in whole breasts were assessed by subgross techniques and significant variations were recorded with age. Parity and laterality were not of significance in these results. Examination of breasts following injection of lactiferous ducts with a radio-opaque medium indicated that more than one subgross lobule type was associated with a single lactiferous duct system.

Comparisons have been made between the radiographic and subgross morphology of slices of breast tissue and results suggest that the parenchymal content of the breast cannot be predicted from radiographic appearances alone.

ACKNOWLEDGEMENTS

My thanks and gratitude are due to many but in particular to the following:

The Faculty of Medicine, University of Edinburgh, for awarding a Leckie-Mactier Fellowship which funded my salary throughout this project and contributed to its technical expenses.

Professor Sir Alastair Currie, for giving me the opportunity to pursue this project and for his advice throughout.

My supervisor, Dr. T.J. Anderson, for teaching me more than I can adequately acknowledge regarding research and who inspired me to complete this thesis.

Miss L. Dunlop, Mr. R. Hogg, Mr. E. Millar and all the staff of the Research Histology Laboratory in the Pathology Department, University of Edinburgh, for their technical advice and assistance.

Mr. J. Waugh for providing and suggesting the use of numerous materials.

Mr. A. Yeoman in the Department of Anatomy for his invaluable aid in the early technical stages of this work.

Mr. I. Gordon, Mr. Renton and Mr. I. Goddard for photographic services.

Mr. I. Lennox for his help with illustration.

Mrs. Carolyn Brown, Miss V. Forbes and Miss K. Andrew for typing with such patience and goodwill.

Mrs. G. Raab from the Medical Computing and Statistics Unit for both explaining and devising statistics, and without whom the analysis of results could not have been presented in the manner in which they appear in the thesis.

The Department of Clinical Surgery and Professor J.K. Mason for unfailing cooperation regarding the provision and obtaining of specimens.

Professor N.M. Gibbs, Mr. P. Jenkins and Mr. R. Trent of the Histopathology Department, St. Luke's Hospital, Guildford, for their technical assistance in processing some breast tissue.

I also wish to thank those people who have offered help both directly and indirectly in the form of encouragement and support when it really counted. These include: Professor J. Boyes, Professor J.C. Southam, Dr. A.A. Shivas, Dr. S. Fletcher, Mr. M. Wilson, Dr. D. Ferguson, Dr. J. Lamb, Mr. J. Gallacher, Dr. A.G. Leitch, Dr. B. Green, Miss C. Cameron, and my family.

CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

The adult breast is a highly modified apocrine sweat gland lying within the subcutaneous tissue of the pectoral region (Fig. 1.1). The organ is composed of epithelial parenchyma and fibrous connective tissue stroma embedded in fat.

The breast is traditionally divided into 12-20 duct systems commonly called lobes. Each duct system has been thought to form a compound tubulo-acinar gland and terminates in a lactiferous duct which opens upon the nipple. The ducts divide many times in the breast to terminate in clusters of lobules. Between 10 and over 100 lobules arise from each lactiferous duct (Vorherr, 1974; Rehman, 1978).

The lobule is the basic structural unit of the mammary gland. Each lobule has a racemose structure and is formed from small blind-ending ductules (Figs. 1.2 & 1.3). The number and size of lobules vary and in general they are largest and most numerous during young womanhood (Bonser, Dossett & Jull, 1961; Haagensen, 1971). They are not distributed evenly throughout the breast (Cooper, 1845; Parkes, 1959; Ingleby & Gershon-Cohen, 1960).

The ductules composing a lobule are lined by a single layer of cuboidal epithelial cells. Additional basal cells found occurring around the ductule are the myoepithelial cells. Each ductule is enveloped by a delicate but well-defined basement membrane. The lobule as a whole is enclosed by a somewhat thicker collagen envelope. Thus the ductular elements of each lobule are embedded within a loose, cellular connective tissue, termed intralobular stroma. Connective tissue between lobules is dense, contains fat

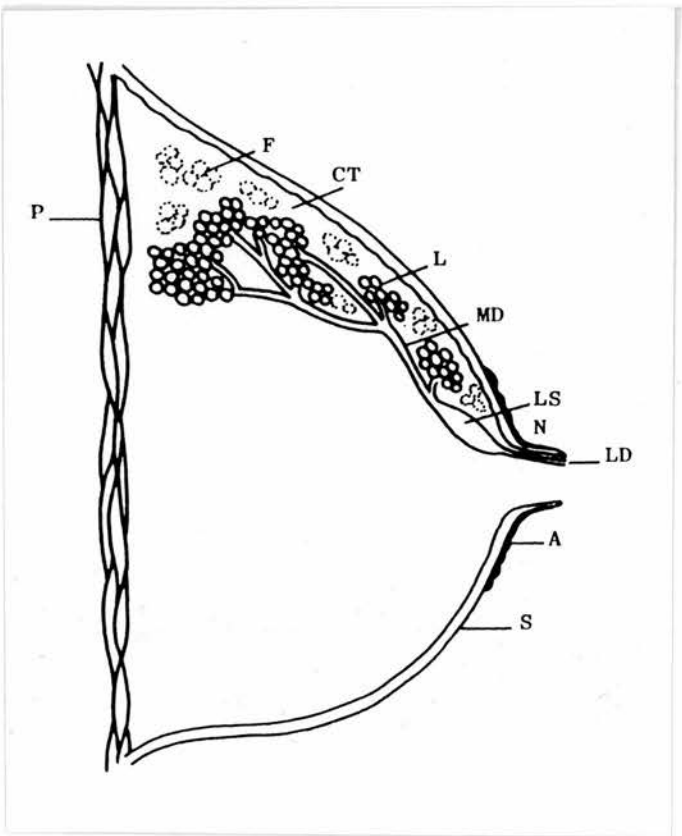

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Fig. 1.1: Diagram of the adult human breast

N - nipple; A - areola; S - skin; LS - lactiferous sinus;

MD - mammary duct; L - clusters of parenchymatous lobules;

CT - fibrous connective tissue; F - adipose tissue;

P - pectoral musculature.

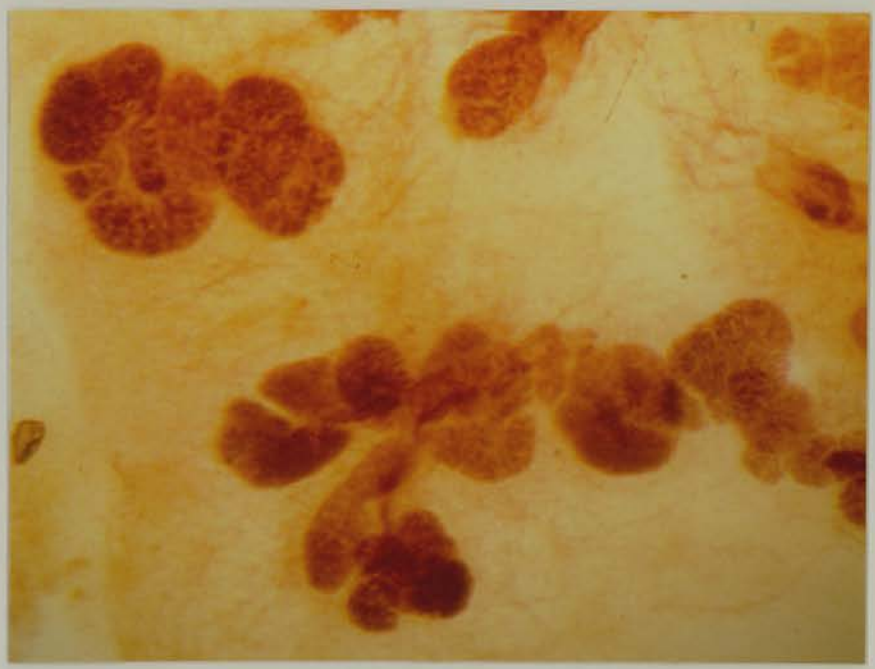
Fig. 1.2

Subgross preparation of normal breast parenchymatous lobules stained with Delafield's haematoxylin and cleared with methyl salicylate (19 years). Mag. x 18.

Fig. 1.3

Histological preparation of fig. 1.2 showing a single parenchymatous lobule composed of ductules lines by cuboidal epithelium and intralobular stroma.

Stained haematoxylin and eosin. Mag. x 120.



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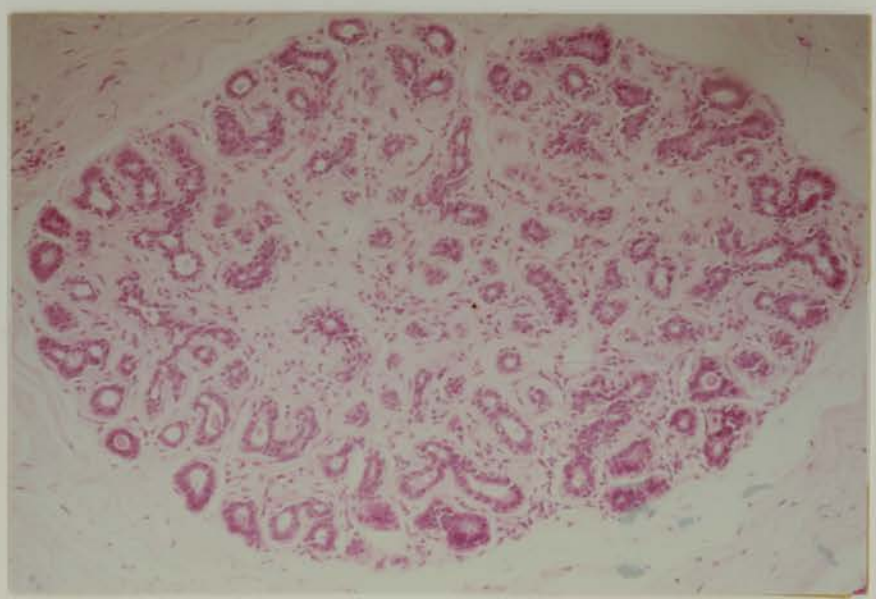

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cells and is termed interlobular stroma.

Embryologically, the breasts develop early in intra-uterine life as an outgrowth of ectoderm from the milk lines, which extend from the axillae to the groin. At birth, and until puberty, breasts of both sexes follow an identical course of development and have a rudimentary parenchymatous structure consisting of a simple system of branching ducts which open on to the nipple. After puberty, the female breasts develop under the influence of sex hormones. The nipple enlarges, the ductal system develops and the formation of lobules takes place (Dawson, 1934; Ingleby & Gershon-Cohen, 1960; Vorherr, 1974).

The changes occurring in the breast from puberty to old age are controlled by ovarian, pituitary and adrenal hormones (Vande Wiele *et al.*, 1970; Chatterton, 1978). The breast tissues mature to their characteristic ductal and lobular structure primarily under the influence of ovarian oestrogens and progesterone. This process of development is supported by other hormones such as insulin, cortisol, thyroid hormone, parathyroid hormone, growth hormone and prolactin (Vorherr, 1974). Oestrogen, progesterone and prolactin undergo cyclic variations, the extent of which is related to age (England, 1974) and may be reflected in the histological appearance of the breast (Bässler, 1970). In the literature many varied accounts describe changes occurring in the breast tissue during successive menstrual cycles. These are discussed in chapter 5.

In pregnancy the mammary gland undergoes changes soon after conception. There is a general hyperplasia of the parenchyma. In

fully developed lobules of pregnancy the number of ductules is increased and they become distended with secretion. It is possible, however, that lobules of pregnancy may contain no more ductules than many of the well-developed lobules seen in the resting breast (Bonser *et al.*, 1961). Regression of the parenchyma takes place after pregnancy and lactation, but the rate of regression is variable and probably incomplete (Berka, 1911; Dawson, 1935a; Dabelow, 1957).

The breast is said to involute at the time of the menopause but, on occasion, involution is thought to precede the menopause by several years (Bonser *et al.*, 1961; Sandison, 1962). Involution does not affect all parts of the breast equally. Very little is known regarding the disappearance of ductules and small ducts in older age groups (Azzopardi, 1979). Frequently, involution proceeds so that one part of the breast may have lost all its lobules and many of its smaller ducts, while another part still retains a normal lobular pattern. When involution is completed, the breast consists of a few major ducts embedded in fat and some fibrous tissue. Involution may be completed some time after the menopause and lobules persisting into old age have been described (Parks, 1959; Wellings, Jensen & Devault, 1976; Wellings, 1980).

Breast cancer is one of the major causes of death in women of the Western world. One out of every 13 women, i.e. 7%, will develop the disease (Leis, 1978). The aetiology and pathogenesis remains unidentified after 75 years of investigation (Brennan, 1980) and breast cancer mortality has not changed in the last 30-40 years. The cause of the disease is unknown and it is therefore not

preventable, but deaths due to late detection can be avoided (Wigle, 1977; Marchant, 1979).

Internationally, the incidence of breast cancer is considered to start at approximately 25 years of age (Byrd, 1978) but 85% of tumours are detected after 40 years of age (Leis, 1978). For a single cancer, that of the breast is the principal killer of women and the now considerable psychological importance of the disease amongst women is aptly reflected in a paper by Small (1978) entitled "A Breast Conscious Society".

Epidemiological studies show that there are several weighting factors in determining the incidence of the disease, amongst which are race, age, body build, parity, ovarian activity and a positive family history of breast disease. According to Leis (1978), breast cancer is not due to a single factor, but to a combination of risk factors including "adverse hormonal milieu" and exposure to carcinogens. The literature describing risk factors has been summarised by Kalache (1981) who states that, while the importance of cancer of the breast is clear, the aetiology of the disease sadly remains obscure.

It has been shown that tumour incidence is higher in nulliparous than parous women (McMahon, Cole & Brown, 1973; Correa, 1975). It has also been shown that an early menarche and a late menopause increase the risk of human breast cancer and further that, in women below 30 years of age, the birth of a first child has the effect of lowering the risk - the younger the mother, the lower the risk (Marchant, 1979). In support of this work, a positive trend of

increasing risk of breast cancer with later ages at first birth was reported by Bain *et al.* (1981). However, Choi *et al.* (1978) have had difficulty in confirming that an early age at menarche is a risk factor, while Miller (1980) maintains that it is by no means certain that age at first pregnancy is an important predisposing factor. He points out that one of the outstanding features of breast cancer is the substantial variation in national incidence and reviews the environmental influences and contribution of diet. Much of the work relating risk factors to nutrition has been performed by de Waard (1975, 1979) and de Waard *et al.* (1974, 1977) who related height and weight to breast cancer incidence.

On an anthropological basis, it is suggested by Short (1974) that there is a simple relationship between the number of menstrual cycles occurring in the reproductive life and cancer incidence; and he postulates that the greater the number of cycles a woman experiences, the greater is the risk of her developing breast cancer.

As a result of the severity of the problem of breast cancer, its epidemiology and the course of the disease, the bulk of research has concentrated on pathological aspects of the breast. Relatively little work has been performed on the normal organ, which remains poorly understood. It would seem axiomatic that a better understanding of the normal would help elucidate the abnormal.

In this project, various aspects of the normal breast have been studied. The subgross architecture of the organ has been examined. The lobules and non-fatty components of whole breast specimens have been quantified. Lobule morphology has been observed and

related to the changing physiology associated with age, parity, the contraceptive pill and the menstrual cycle. The relationship between lobule quantity, lobule morphology and radiographic appearances has been investigated. The incidence of mucopolysaccharides within ductules and in the intralobular stroma of the breast has been related to menstrual cycle status.

CHAPTER 2

SUBGROSS EXAMINATION OF BREAST STRUCTURES

INTRODUCTION

When literature describing the normal breast is examined in detail, a confused pattern emerges. Definitions of normal gross breast structure vary considerably. Ingleby & Gershon-Cohen (1960), when describing the composition of the adult breast, state:

> Before embarking on a description of the normal breast, certain terms in general use require clarification. Such words as trabecula, stroma, parenchyma and lobule are defined in books, but when applied to the breast, there is considerable difficulty knowing just what is meant, a difficulty that most authors do not face squarely.

The same problem relating to the microanatomy of the breast is described by Azzopardi (1979) who mentions that the variation occurring in terminology would matter less if the terms employed were clearly defined.

In spite of detailed histological descriptions of ductal and lobular development and structure in the literature (Cheatle & Cutler, 1931; Dawson, 1934; Geschickter, 1945; Foote & Stewart, 1945a; Dickson & Hewer, L950; Ingleby & Gershon-Cohen, 1960; Bonser *et al.*, 1961; Cutler, 1962; Vorherr, 1974), no authors have described in any detail the morphology of duct branching systems or the distribution and quantity of lobules in the human breast. Dawson (1934) begins her series of articles on the normal breast by stating that most writers on the pathology of the breast assume a knowledge of its normal microscopic anatomy and physiology. A parallel situation seems to exist for the macroscopic anatomy of the normal human breast and it seems fair to suggest that there is today a

greater understanding of the pathological than the normal organ.

Standard anatomical texts divide the tissue of the breast into 15 to 20 lobes, each being separated by fibrous tissue septae. They describe the lobes as pyramidal in shape and grouped in a radiating fashion around the nipple. Surgical text books (McGregor & Du Plessis, 1969; Rush, 1974; Wilson, R.E., 1981) and volumes dealing solely with aspects of the breast (Ingleby & Gershon-Cohen, 1960; Bonser *et al.*, 1961; Haagensen, 1971) give similar accounts of gross breast structure, describing the distribution of breast tissue in terms of lobes, segments or compartments. In none of the books is the gross morphology of ducts and lobules considered further before attention is focused on histological descriptions of the breast parenchyma.

In contrast is the work of Sir Astley Cooper in 1845 who, after injecting milk ducts with wax and then boiling and macerating the specimen, was able to examine the whole breast as a gross specimen in three dimensions. He described the distribution of ducts in the breast in detail and some of his observations are summarised as follows: The margins of the breast do not form a regular disc and the branching ducts do not radiate equally to the circumference; some are longer than others and become lost in the fascia encircling the breast. Not all the ducts terminate at the edge of the breast, but are "turned upon" the gland to form a "hem" at the circumference. On the anterior surface of the breast many ducts are turned forwards to the skin and are connected to it by fibrous "ligamenta suspensoria". The "milk tubes" (i.e. major ducts) divide into branches which increase in number from the centre to the circumference, the radiation of one "tube" occupying one-fifth to one-sixth

of the breast circumference. The breast is not formed into regular lobes by ramification of the ducts, because the ducts ramify and intermix with each other and thus the simplicity and uniformity of their divisions is destroyed: "like the roots of a tree, one root growing between others and destroying the regularity and distinctness of growth like fingers intertwined".

By the use of different coloured wax injections, Cooper concluded that mammary ducts do not communicate with each other. Only one incidence of communication was seen in 200 cases. He speculated whether this occurrence might have been an accident, adding that - even if not - the phenomenon must be uncommon. That ducts do not communicate had been noted in animals. Cooper also observed that dilations occur when two or three branches enter a duct, both during and after lactation.

Therefore, Cooper directly contradicts the statements made in the majority of texts of today regarding gross breast structure, many of the authors and editors of which refer to the "ligamenta suspensoria of Cooper" and must therefore have been aware of his work.

Stiles (1892) criticised methods of studying the breast which involved dissection with or without boiling and partial maceration as neither convenient nor satisfactory. He stated that the parenchyma or gland tissue proper is so intimately connected with the fibrous framework or stroma that the two cannot be separated. He used nitric acid to render all the breast tissue except the fat an opaque white. By subsequently washing in water, the connective

tissue became translucent and gelatinous whereas the parenchyma remained opaque. The fat was unaltered. He maintained that such a method differentiated the various constituents in an undisturbed relation so that the form, extent and arrangement could be accurately defined. Stiles then described the anatomy of the breast from a surgical point of view, but did not mention that ducts radiate out from the nipple like the spokes of a wheel, nor that there are fibrous tissue septae between lobes. He described lobules as being aggregated into grape-like clusters of varying size, forming larger compound lobules. These were then collected into lobes by the branches of a lactiferous duct.

The ideas of Cooper and Stiles are reflected, in part, in a minority of volumes. Bruce, Walmsley & Ross (1964) present the following definition of the normal breast:

> The main duct and its lateral and terminal branches (ductules) clothed in fat constitutes a mammary lobe, which consists of a series of lobules comprising the several sub-branchings. The lobes are intimately related to one another and cannot be separated by dissection.

Cutler (1962) states that there is:

> considerable overlapping of the peripheral lobules, so that although it is often mentioned that each of the 15-20 ducts collects the secretion from a single lobe, the individual lobes are usually not discrete.

Bailey (1977) states:-

> The lobule is the basic structural unit of the mammary gland. From 10 to 200 lobules empty by means of ductules into a lactiferous duct of which there are from 15-20.

Dickson & Hewer (1950) maintain that the gross appearance of the breast does:

> not reflect the lobar plan; the parenchymatous parts are embedded in a stroma of connective tissue which gives no external evidence of the limits of the lobes.

It is apparent that there is some disagreement as to how the duct branching systems in the breast are arranged. There are no studies in the literature which describe the patterns of ramification of ducts in the adult human breast. Dawson (1935a) makes some comment on duct development in the foetus. She states that, when dichotomy occurs in a developing duct, the growing tip forms two centres of growth and from each a new segment of duct develops. Growth then proceeds with the formation of the same type of structure, but the new segments are shorter in length and "apparently" narrower in calibre. "This diminution in size and length seems progressive with each new division, though there are probably many variations in the form of structure laid down."

Tanaka & Oota (1970), after examination of breast specimens in three dimensions following the use of a staining and clearing technique, state that the main lactiferous ducts branch with organised dichotomous divisions until they terminate in ductules which form lobules. Wellings (1980) also describes ducts branching dichotomously, eventually to form terminal ductal lobular units (Wellings, Jensen & Marcum, 1975). Generally it has been an assumption, often unwritten, that once growth and development are completed, the ducts in the adult resting breast are branching in a dichotomous manner towards the periphery, becoming smaller in calibre and terminating in lobules.

For this research project, a method was adapted to examine the whole breast in a three dimensional manner by staining and chemically clearing thick sections of breast tissue. In recent years interest has revived in the use of thick sections of tissue. Indeed, this was the original method for examining material before the invention of the microtome, when sections were cut freehand.

The thickness of large tissue sections can vary depending on the techniques selected. Chesterman & Leach (1949), Gough & Wentworth (1949), Parks (1959), Gallager & Martin (1969) and Schwarz & Sooneung (1969) are amongst authors working on both animal and human material who have made use of whole mount sections of approximately 6-200 μm thickness. However, it is not possible to examine breast specimens in their entirety by employing such thinly sliced sections. Therefore, the method adopted in this project for processing whole breasts makes use of 2 mm thick sections of tissue. The most fundamental differences between this method for examination of large slices of breast tissue and similar methods which have previously been described (Stiles, 1892; Marcum & Wellings, 1969; Tanaka & Oota, 1970; Cameron & Faulkin, 1974; Migliori, 1975; Sarnelli, Sabò & Squartini, 1980a; Sarnelli *et al.*, 1980b) are, first, the coronal as opposed to sagittal slicing of the breast specimens and, second, the use of Delafield's haematoxylin as the staining agent. Coronal slicing of tissue has been employed before but to a limited extent in the work described by Davies, Roberts & Richardson (1973).

In this chapter the method adopted in the study for processing whole breast specimens for subgross stereomicroscopic examination is

described. Observations are made on the distribution of the breast parenchyma and the patterns of branching of the ducts in their passage from the nipple to the periphery of the breast.

MATERIALS

Whole breasts were obtained from autopsy suites and operating theatres. The majority of female subjects at autopsy are post-menopausal, and most premenopausal breasts had to be obtained from surgical excisions (mastectomies). A total of 50 breasts have been processed during the course of this research project. The complete listing of these with relevant case details is provided in Appendix C, Tables 1-3.

METHODS

On receiving a mastectomy or autopsy of breast, the dimensions, i.e. mammary fat pad and skin ellipse, were recorded. The upper outer quadrant (UOQ) and the 12 o'clock position were marked with both plastic tags and Indian ink to maintain correct orientation throughout processing. The breast was radiographed prior to fixation using a Faxitron 43805 radiographic system (Hewlett-Packard, Berks., England) at a 2½ minute, 25 kvp (anode tube voltage) exposure. This radiograph provided a useful record of correct orientation prior to processing.

The breast was then pinned down to a cork board which was inserted in a plastic tub of 4% formaldehyde in phosphate buffer fixative (Carson, Martin & Lynn, 1973) for 72 hours. The fixative was changed twice in the first 24 hours and then after each subsequent 24 hours. Attachment to the cork board maintained the

breast in a good shape and avoided any curling at the edges of the specimen during fixation. After 72 hours the breast was removed from the fixative and cork board, rinsed for 2 hours in warm running tap water and then for 4 hours in cold water to wash away the fixative from the surface. The specimen was immersed in cold water and then placed in a drying cabinet at 37^0C overnight.

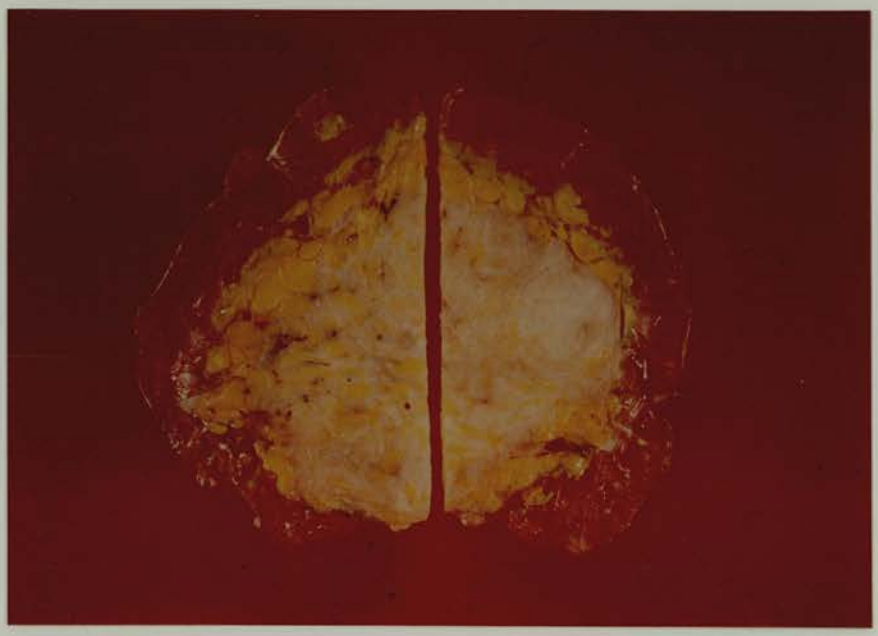
The next day the breast was removed from the water and dried. A line was drawn with Indian ink to join the 12 and 6 o'clock positions and the UOQ was re-marked. The breast was inverted and lowered into a dry plastic tub containing heated 12.5% gelatin (BDH Ltd., Poole, England). The tub was placed in a 4^0C refrigerator for 4 hours to allow the gelatin to set. After this period the gelatin was removed from the tub as an intact block and excess trimmed to approximately 2 cm around the breast. The Indian ink markings maintaining orientation of the specimen were clearly visible through the gelatin. The breast was then ready for slicing using a commercial Excel-Boston 250 mm gravity slicing machine (Excel Equipment Ltd., Chester, England). Specimens processed for the preliminary study were cut along the line from 12 to 6 o'clock and the two halves sliced separately. Thereafter, following alteration of the quantitation techniques, breasts which could be accommodated on the machine were sliced whole. Larger specimens were bisected as described (Fig. 2.1). The machine was set so as to give 2 mm slices in a coronal plane. To provide direct comparison of coronal with sagittal slicing, when a pair of breasts was available, one was sliced in the sagittal plane. The first two and the last two slices to come off the slicer from each specimen were discarded.

Fig. 2.1

Coronal slices of breast tissue bisected in a sagittal plane (41 years). The clear gelatin used for embedding and supporting the breast during sectioning may be seen around the periphery of the tissue. (Mag. x 0.4).

Fig. 2.2

Coronal slice of breast tissue (29 years). The white tissue consisting of fibrous tissue and parenchyma is clearly visible against a background of yellow fat. The gelatin used for embedding has been washed away from the tissue. The metal identification tag at the 12 o'clock position marks the reference number of the breast and should be disregarded in all photographs. (Mag. x 0.5).



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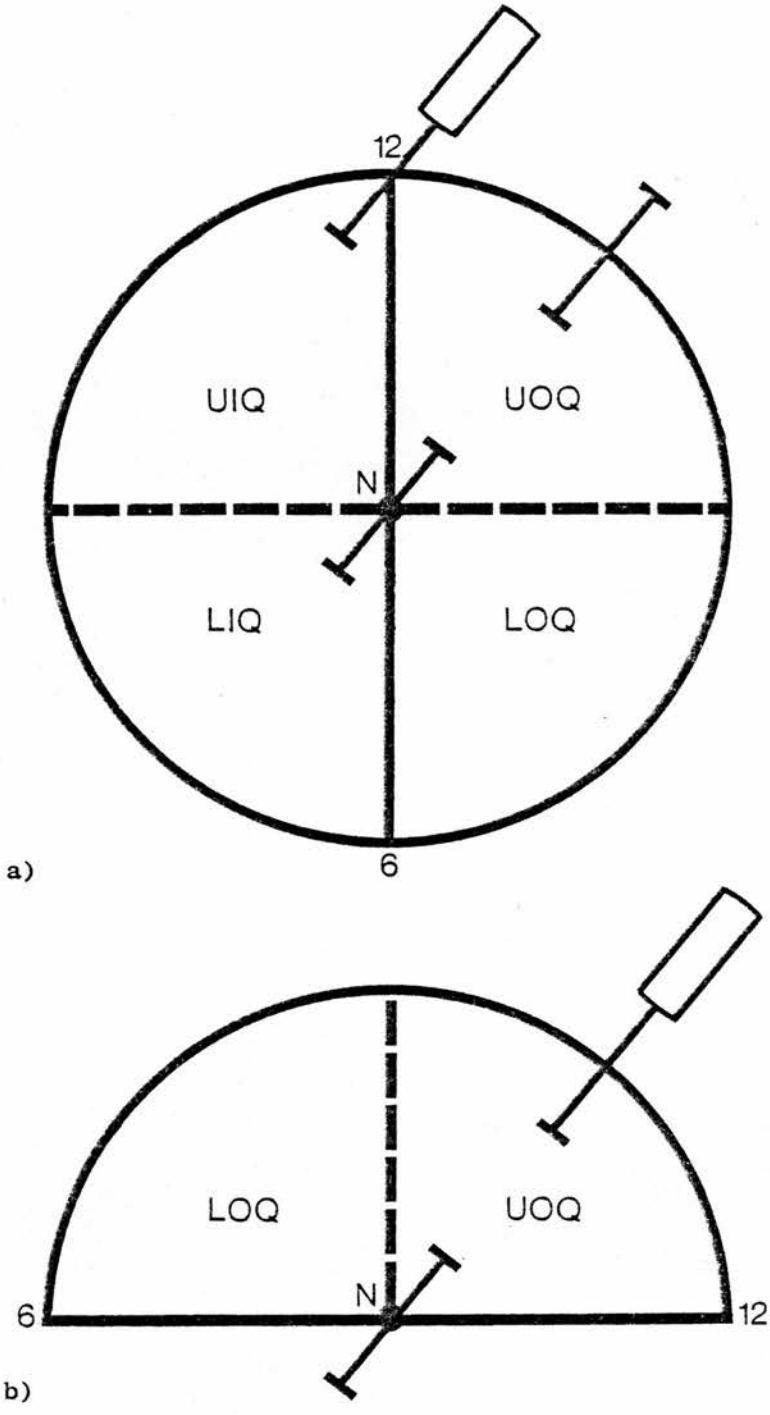


a) Coronal slice of whole breast.
b) Coronal slice of whole breast bisected in a sagittal plane.

N	= nipple
UOQ	= upper outer quadrant
UIQ	= upper inner quadrant
LOQ	= lower outer quadrant
LIQ	= lower inner quadrant
6 and 12	= o'clock positions

= identification tags

Fig. 2.3 ORIENTATION OF BREAST SLICES.

These slices were incomplete and predominantly composed of gelatin. Staining and clearing of the discarded tissue showed almost entirely fat. Any parenchymal or fibrous tissue component was negligible.

After labelling the slices, the gelatin was washed out under hot tap water (Fig. 2.2) and the tissue left to continue fixation overnight in Carson's fixative. Each slice was labelled with a plastic tag to maintain orientation relative to the nipple and a further plastic tag with a metal label (Dymo Ltd., Feltham, England) attached denoting side of breast, identification of breast and slice number at the 12 o'clock position. Whole breast slices had an additional plastic tag placed in the UOQ to avoid accidental inversion during handling and processing. Thus, the breast was orientated so that the upper outer quadrant (UOQ), the upper inner quadrant (UIQ), the lower outer quadrant (LOQ), and the lower inner quadrant (LIQ) could be differentiated (Fig. 2.3). Unless embedding, slicing, labelling and washing out of gelatin is achieved in one day, there is a danger that a longer contact with the breast tissue may result in formalin not washed away from the surface rendering the gelatin insoluble. This, then, becomes hard and brittle during subsequent processing and distorts the slices. After overnight fixation, the slices were rinsed for 15 minutes in cold running tap water, dried with paper towels and radiographs were taken using an appropriate exposure (Appendix A, VII). The slices were then returned for a final 24 hours in fixative.

Fixation completed, the following staining and dehydration schedule was used:

1. Wash 30 minutes in cold running tap water.
2. Stain 45 minutes in 50% Delafield's haematoxylin with frequent agitation.
3. Wash 30 minutes in cold running tap water.
4. Decolourise in acid ethanol (2% hydrochloric acid/ethanol).
5. Differentiate by washing in running tap water 2-4 hours.
6. Dehydrate in 50% ethanol overnight.
7. Dehydrate in 95% ethanol 12 hours.
8. Dehydrate in 99.8% ethanol overnight.
9. Dehydrate in 99.8% ethanol 12 hours.
10. Dehydrate in 100% ethanol overnight.
11. Clear in methyl salicylate 6-12 hours.

When staining has been completed, the parenchymatous tissue is seen as red or purple against the unstained connective tissue background (Fig. 2.4). After clearing in methyl salicylate, the connective tissue becomes largely transparent, thus improving the contrast between the connective tissue and the parenchyma (Fig. 2.5).

The method adopted in this project is a modification of a similar procedure used by Wellings *et al.* (1975) who in turn largely developed their method from the work of Ingleby & Holly (1939). Experimentation undertaken in the development of the technique used in this study is described in Appendix A, I-VII.

Axillary Tail Tissue

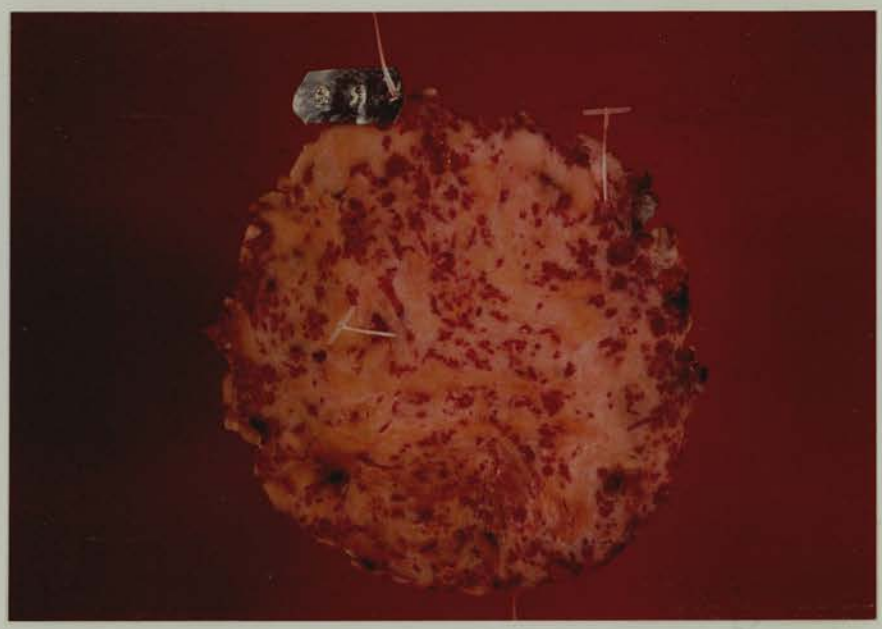
Axillary tail tissue, when received with a mastectomy as one or more specimen, was measured and examined for lymph nodes which, if present, were removed for diagnostic purposes. Any residual

Fig. 2.4

Coronal slice of breast tissue (56 years). The red stained parenchymatous tissue consisting of breast ducts and lobules may be clearly distinguished from the background fibrous tissue and fat.
Delafield's haematoxylin. (Mag. x 0.5).

Fig. 2.5

Field of cleared breast tissue (21 years). A terminal duct with its attached parenchymatous lobule may be seen branching from a larger duct.
Delafield's haematoxylin. (Mag. x 25).



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tissue was put through the slicing, staining and clearing procedures as described but without gelatin embedding.

Biopsy Sites

Details of the sizes and sites of all biopsies taken prior to mastectomy were obtained from case histories (Appendix C, Tables 1 & 3). Any apparent residual tumour and its position within the breast was also noted; its presence or absence was confirmed by microscopic examination following processing.

Photography

Black and white photographs enlarged to scale were taken of each slice of breast tissue to provide an easily accessible record of every specimen. Colour photography was also performed to illustrate the parenchyma, particularly at higher magnifications. A better contrast of the parenchymatous structures against the connective tissue background is obtained using colour photography which was therefore employed as much as costs allowed. Details of all film types and equipment employed are provided in Appendix A, XIV.

Duct Cannulation

Duct injections were performed on 10 whole breast specimens obtained from surgical mastectomy. The technique is described in full in Appendix A, VIII. Briefly, breast ducts were cannulated at the nipple and manually injected with micropaque (Nicholas Labs., Slough, Berks.). The breast specimens were thereafter radiographed

whole, both in a coronal plane and using a 45^0 angle of tilt to the horizontal. The number of lactiferous ducts injected in any one of the 10 specimens varied between 1 and 8 ducts. After each duct was injected, the specimen was radiographed to obtain an impression of the morphology of the duct systems relative to each other. Details of the radiographic exposures are provided in Appendix A, VII. On completion of this process, the breasts were sliced, stained and cleared in the manner already described.

Examination of Tissue

Processed slices of breast tissue were examined by a Wild-Heerbrugg M8 zoom stereomicroscope (Leitz Ltd., Luton, England), having a range of x 6 to x 50 magnification. To facilitate examination of the tissue slices, they were either submerged in a glass dish filled with methyl salicylate, or heat sealed in transparent bags (Kapak Corporation, Bloomington, USA).

RESULTS

The results are presented below in a series of photographs. The first set (Figs. 2.6-2.15) show coronal slices of cleared breast tissue which were selected from comparable depths in the breast. It should be noted that almost all the ducts and parenchymatous lobules lie in fibrous tissue which is cleared substantially less by methyl salicylate than adipose tissue. In the majority of illustrations of whole slices, the boundaries of the fibrous tissue are usually identifiable. However, the extent of clearing of the fibrous tissue varies from breast to breast and is not readily quantifiable. The photographs show the differences in parenchymal distribution and quantity in breasts as well as the variation between breasts.

The second set of photographs (Figs. 2.16-2.41) are fields of cleared breast tissue at varying magnifications. When viewed in isolation and out of the context of the individual breasts from which they were derived, there appear to be discrepancies in magnification between fields. Ducts vary in size according to their status in the breast and were magnified to the limit permitted by the photographic field. Likewise, lobules varied in size from one breast to another and, to a more limited extent, within individual breasts. The magnification of structures in one photograph therefore is not directly comparable with that in another. The object of the second group of illustrations was to investigate the duct branching patterns in the human breast for comparison with those found in animals.

The final set of photographs (Figs. 2.42-2.53) are from radiographs and cleared tissue derived from breasts which underwent duct injection procedures. The aim of this section of the study was to supplement and, where possible, confirm the observations made on uninjected cleared tissue and to ascertain whether the division of breasts into lobes occurs.

Fig. 2.6

Coronal slice of cleared breast tissue (43 years). The parenchyma is dense, well circumscribed and compactly arranged. Delafield's haematoxylin. (cm scale top right).

Fig. 2.7

Coronal slice of cleared breast tissue (38 years). The parenchyma is dense and confined to the lower half of the slice of tissue. Delafield's haematoxylin. (cm scale top right).

2·6

2·7

Fig. 2.8

Coronal slice of cleared breast tissue (30 years). The parenchyma is scanty and largely confined to the upper quadrants.

Delafield's haematoxylin. (Mag. x 0.6).

Fig. 2.9

Coronal slice of cleared breast tissue (56 years). The parenchyma is most heavily concentrated in the upper and outer portions of the slice of tissue.

Delafield's haematoxylin. (cm scale top right).

2·8

2·9

Fig. 2.10

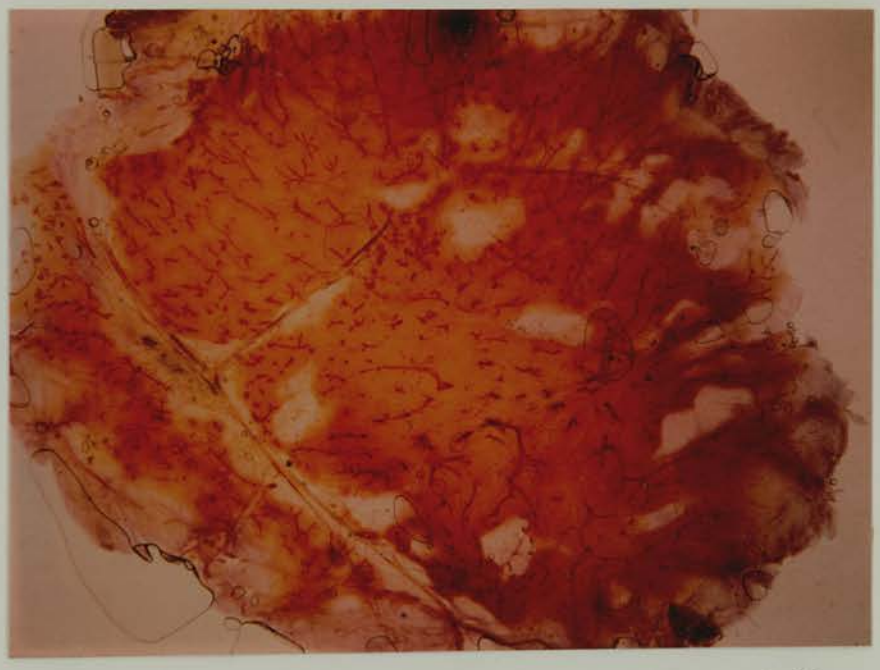
Coronal slice of cleared breast tissue (21 years). The parenchyma, which is predominantly composed of ducts, is evenly distributed throughout the tissue.

Delafield's haematoxylin. (Mag. x 0.6).

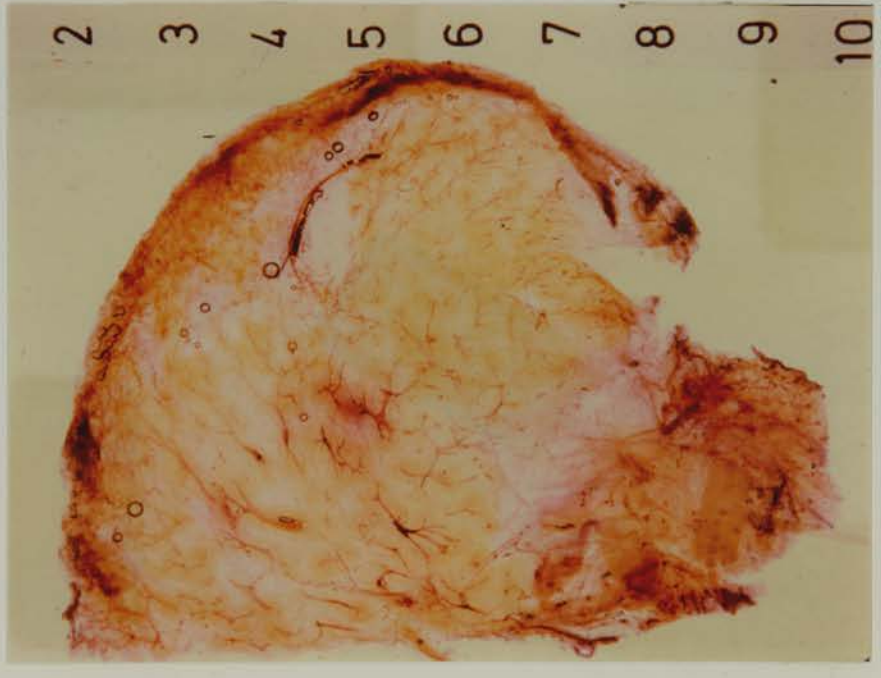
Fig. 2.11

Coronal slice of cleared breast tissue bisected in a sagittal plane to show the inner quadrants (46 years). The parenchyma, which is distributed throughout the slice, is composed predominantly of ducts.

Delafield's haematoxylin. (cm scale at top).



2·10



2·11

Fig. 2.12

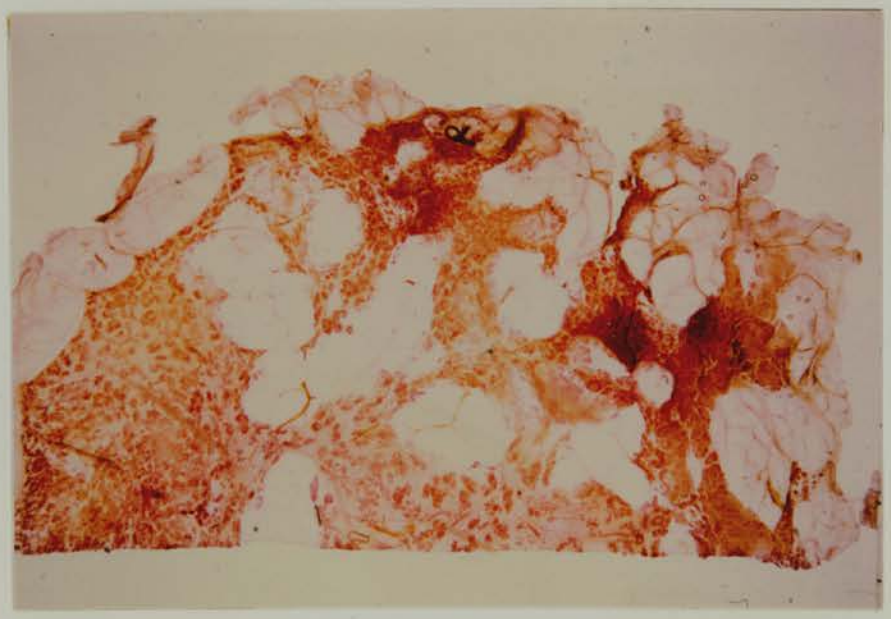
Coronal slice of cleared breast tissue bisected in a sagittal plane to show the outer quadrants (25 years). The parenchyma has an extensive distribution throughout the slice and fat lobules are clearly circumscribed.

Delafield's haematoxylin. (Mag. x 0.7).

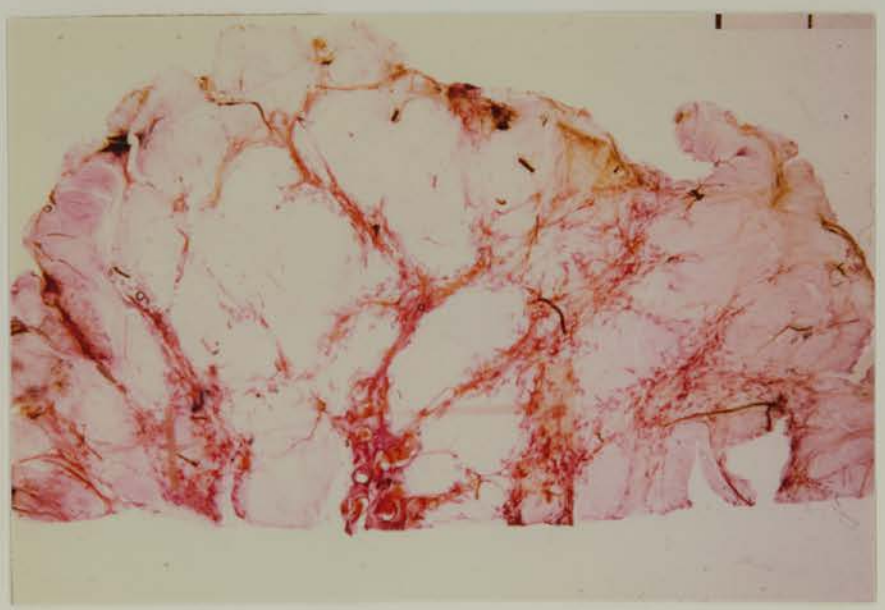
Fig. 2.13

Coronal slice of cleared breast tissue bisected in a sagittal plane to show the inner quadrants (46 years). The parenchyma is largely confined to fibrous tissue septae which pass between fat lobules.

Delafield's haematoxylin. (cm scale top right).



2·12



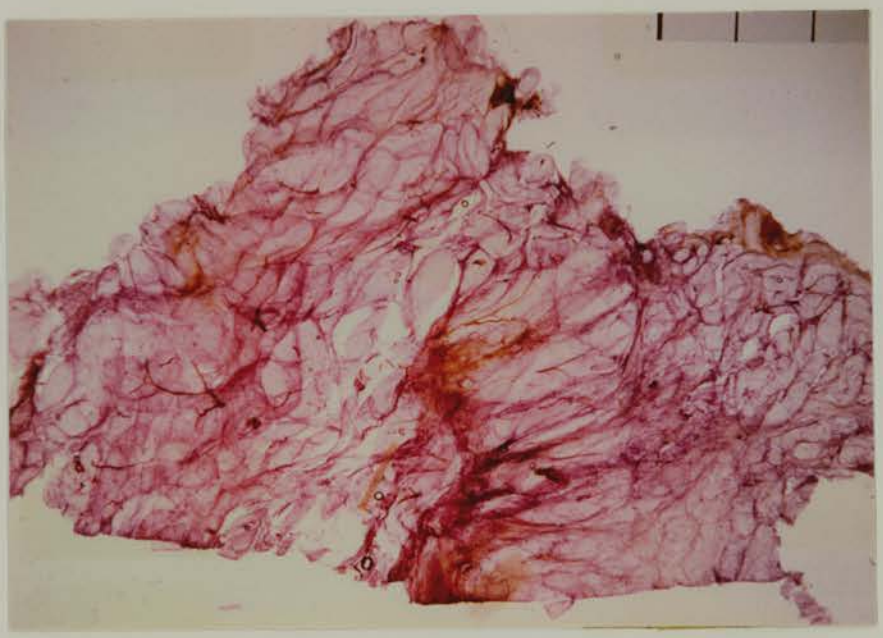
2·13

Fig. 2.14

Coronal slice of cleared breast tissue bisected in a sagittal plane to show the outer quadrants (57 years). There is very little parenchyma, the majority of the tissue being fat.
Delafield's haematoxylin. (cm scale top right).

Fig. 2.15

Coronal slice of cleared breast tissue - duct injection study (29 years). The superficial half of the nipple which has been bisected and folded over gives rise to the dense opacity (lower field right). The proximity of parenchymatous lobules to the nipple is clearly demonstrated.
Delafield's haematoxylin. (cm scale top right).



2·14

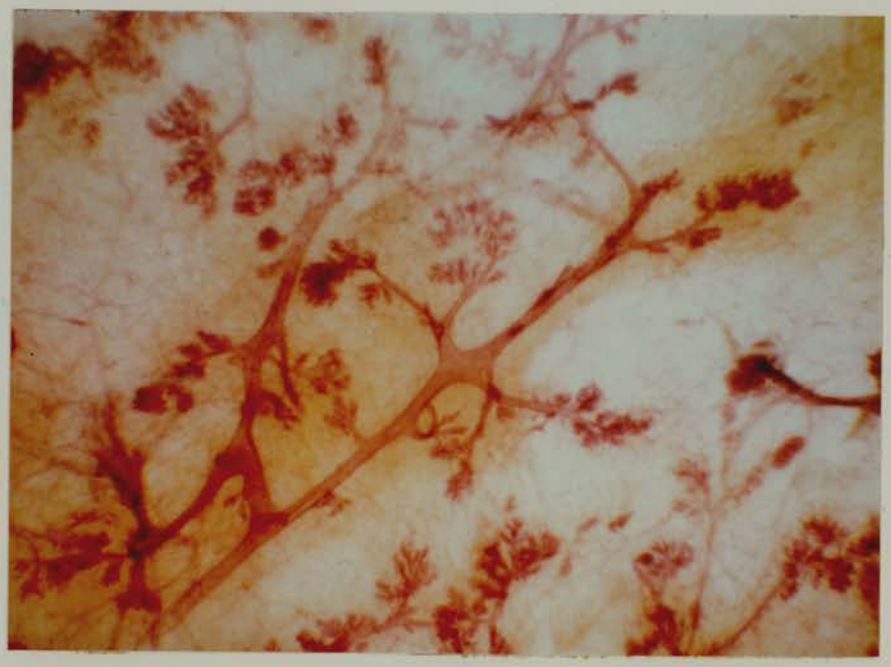
2·15

Fig. 2.16

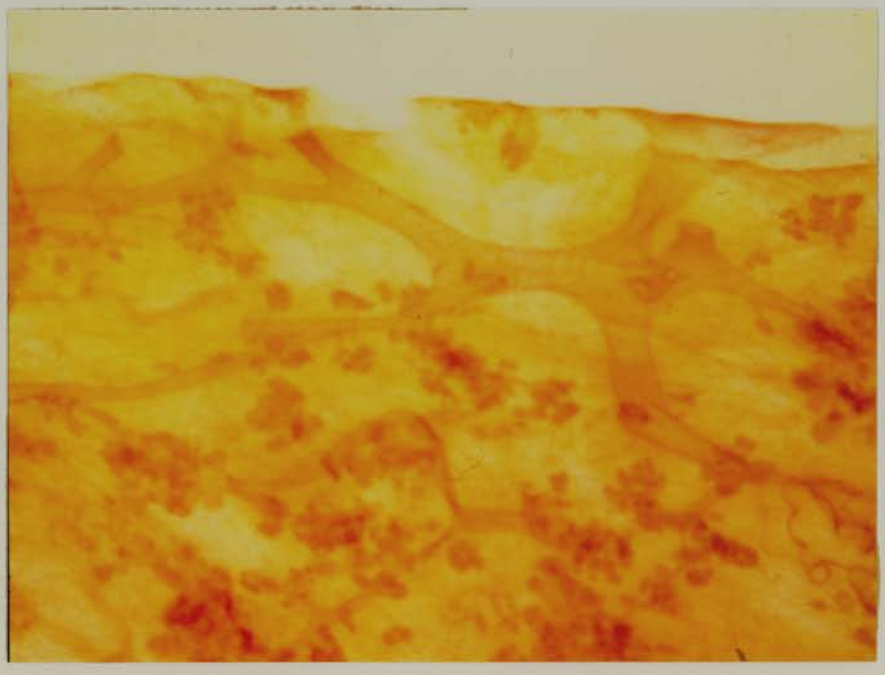
Field of cleared breast tissue (19 years). The ducts and parenchymatous lobules are clearly seen stained red contrasting with the cleared background of fibrous tissue and fat.
Delafield's haematoxylin. (Mag. x 9).

Fig. 2.17

Field of cleared breast tissue (41 years). At the top of the picture sizeable ducts may be seen terminating abruptly at the periphery of the tissue.
Delafield's haematoxylin. (Mag. x 6).



2·16



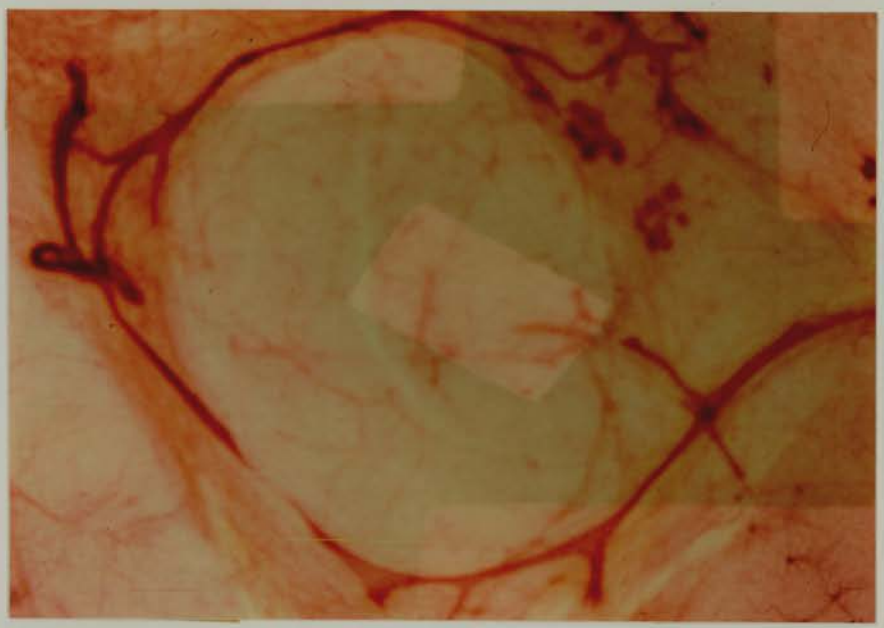
2·17

Fig. 2.18

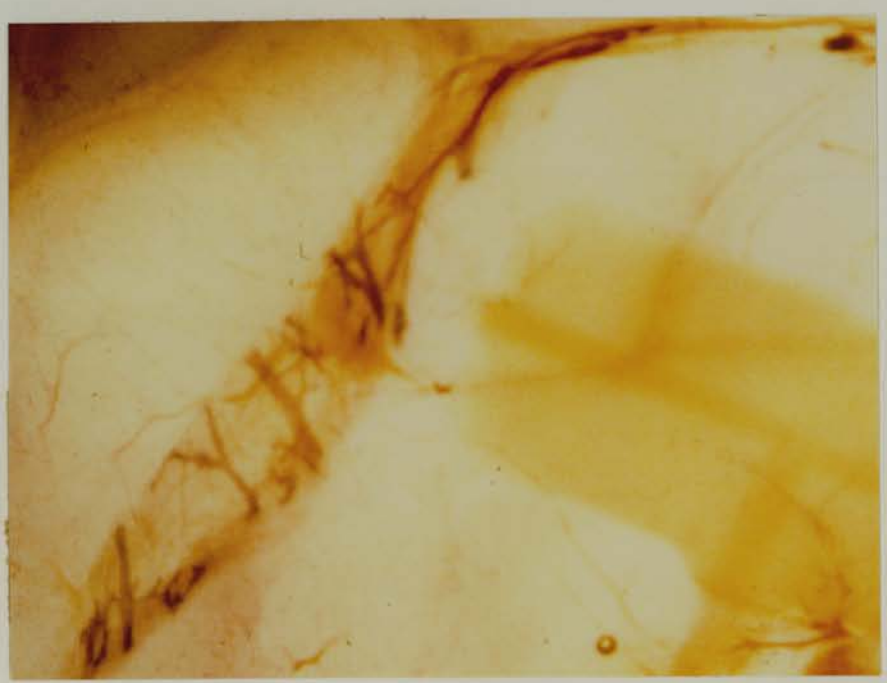
Field of cleared breast tissue (50 years). A duct enters mid-field right and encircles a fat lobule terminating in a cluster of parenchymatous lobules.
Delafield's haematoxylin. (Mag. x 6).

Fig. 2.19

Field of cleared breast tissue (57 years). On the right of the picture an enlargement of a plastic identification tag may be seen superimposed on the tissue. On the left, ducts may be observed travelling along a septum of fibrous tissue dividing two fat lobules.
Delafield's haematoxylin. (Mag. x 12).



2·18



2·19

Fig. 2.20

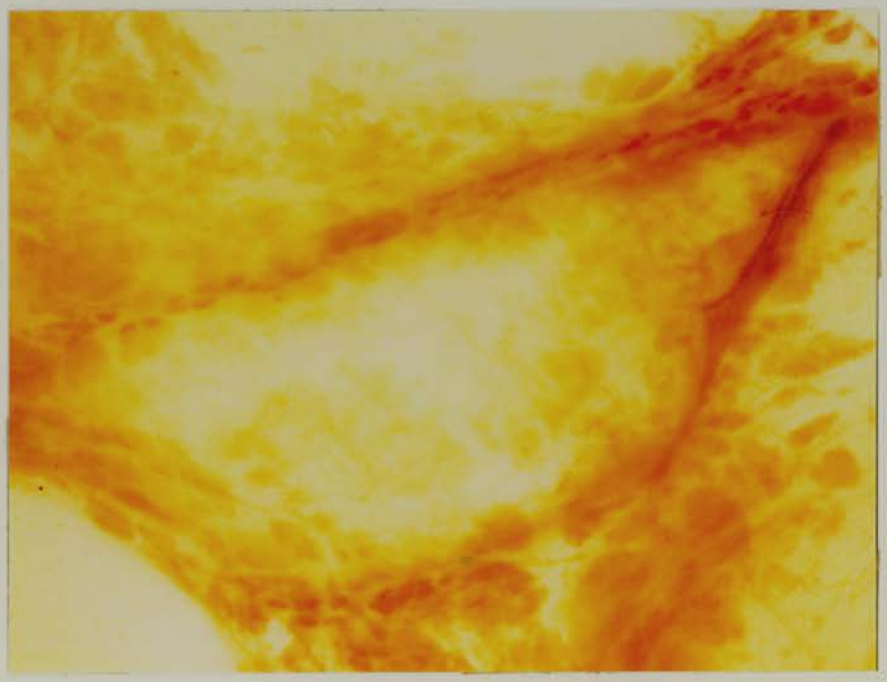
Cooper's ligaments of the breast. Reproduced from Cooper (1945).

Fig. 2.21

Field of cleared breast tissue (31 years). This shows parenchymatous lobules largely confined to the fibrous septa around fat lobules.

Delafield's haematoxylin. (Mag. x 6).

2·20



2·21

Fig. 2.22

Coronal slice of cleared breast tissue bisected in a sagittal plane to show the outer quadrants (26 years). The parenchymatous elements are distributed in a whorled type of pattern.

Delafield's haematoxylin. (cm scale top left).

Fig. 2.23

Field of cleared breast tissue (35 years). A duct entering top right may be seen to double back in its course to leave the field bottom right.

Delafield's haematoxylin. (Mag. x 12).

2·22

2·23

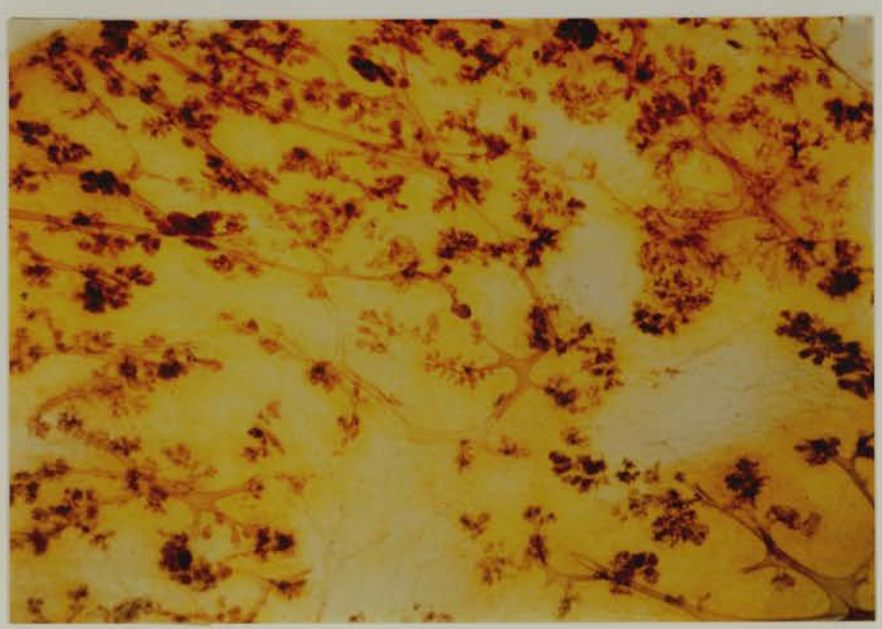
Fig. 2.24

Field of cleared breast tissue (55 years). A duct may be seen to turn back upon itself in its course through the tissue and also to undergo marked alterations in its calibre.
Delafield's haematoxylin. (Mag. x 12).

Fig. 2.25

Field of cleared breast tissue (19 years). The parenchyma consisting of ducts and lobules is seen in contrast to the background fibrous tissue and fat. In the outlined area of the field the ducts do not branch in a dichotomous fashion. An enlargement of this area is illustrated in the figure which follows.
Delafield's haematoxylin. (Mag. x 6).

2·24



2·25

Fig. 2.26

Fields of cleared breast tissue which are enlargements of the area outlined in Fig. 2.25 (19 years). The ducts do not divide dichotomously but branch in an apparently random manner to form a reticulum or network-like structure.

Delafield's haematoxylin. (Mag. x 14).

2·26

Fig. 2.27

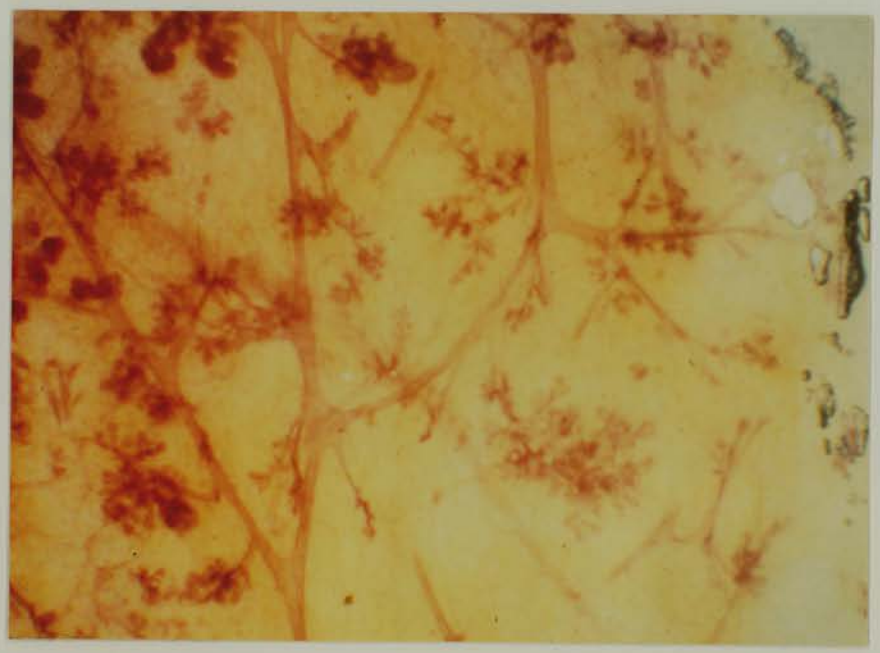
Field of cleared breast tissue (19 years). A duct in the centre of the field is connecting two ducts of greater calibre. The connected ducts have the visual effect of travelling in opposite directions.

Delafield's haematoxylin. (Mag. x 9).

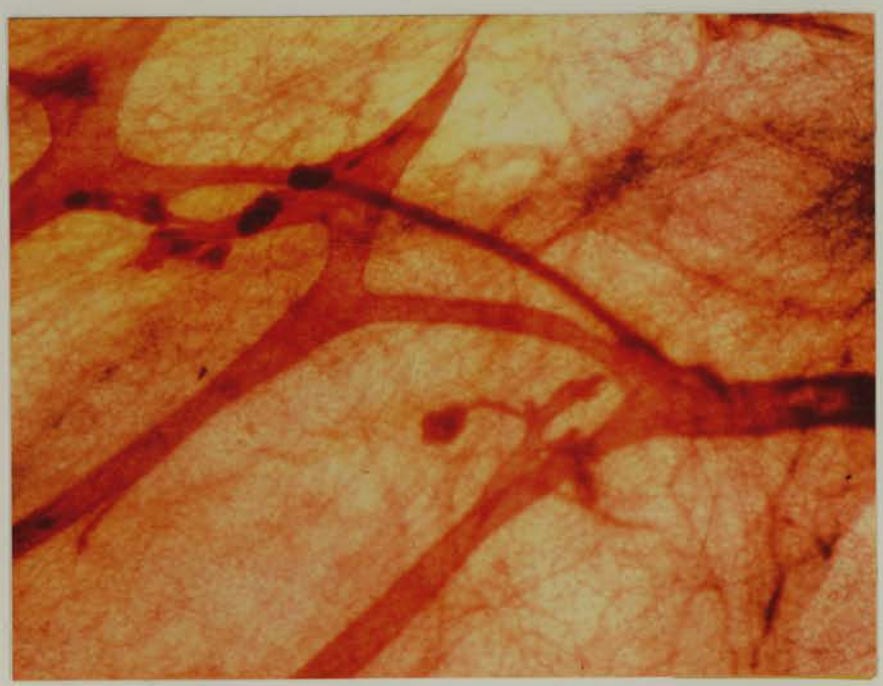
Fig. 2.28

Field of cleared breast tissue (31 years). A duct enters mid-field right and branches in an arcading manner. It is evidently connected to the duct at top left.

Delafield's haematoxylin. (Mag. x 14).



2·27



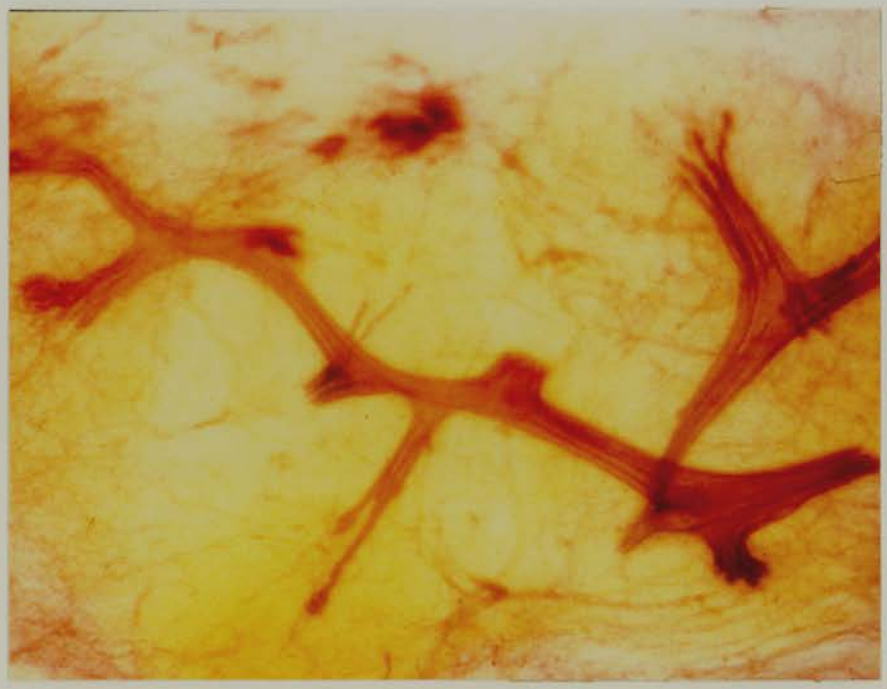
2·28

Fig. 2.29

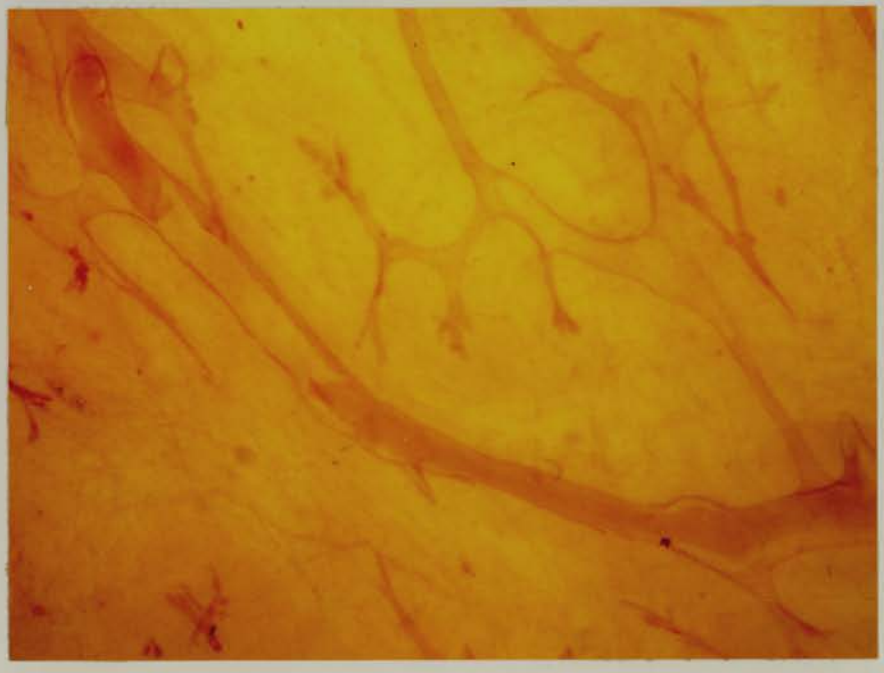
Field of cleared breast tissue (35 years). The duct entering the field bottom right displays an arcading pattern of branching. Delafield's haematoxylin. (Mag. x 12).

Fig. 2.30

Field of cleared breast tissue (46 years). On the right, a duct of small calibre connects ducts of larger calibre. In the centre of the field an arcading pattern of duct branching is seen. Delafield's haematoxylin. (Mag. x 6).



2·29



2·30

Fig. 2.31

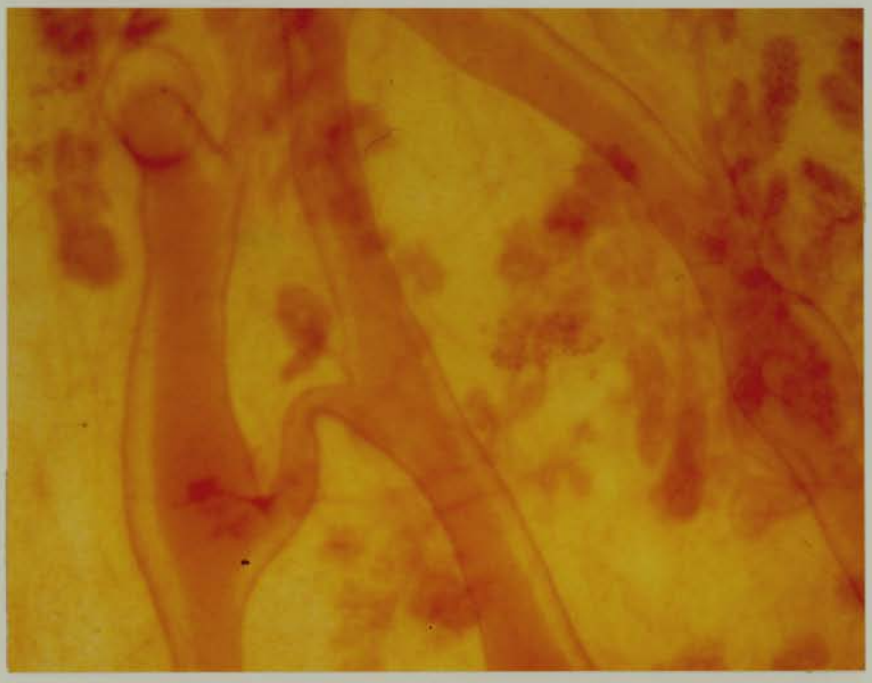
Field of cleared breast tissue (41 years). In the centre and to the left of the field, two ducts are connected by a third of smaller calibre.

Delafield's haematoxylin. (Mag. x 14).

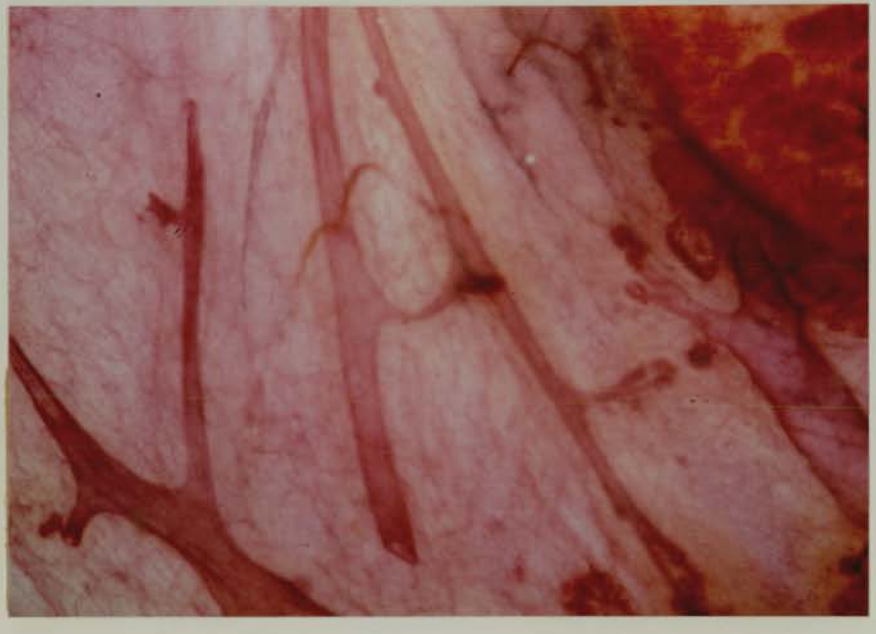
Fig. 2.32

Field of cleared breast tissue (38 years). Two ducts enter from the top of the field and in the centre of the field are joined by one of smaller calibre.

Delafield's haematoxylin. (Mag. x 6).



2·31



2·32

Fig. 2.33

Field of cleared breast tissue (35 years). Dilations of the lumen are seen where more than two ducts are joined.

Delafield's haematoxylin. (Mag. x 6).

Fig. 2.34

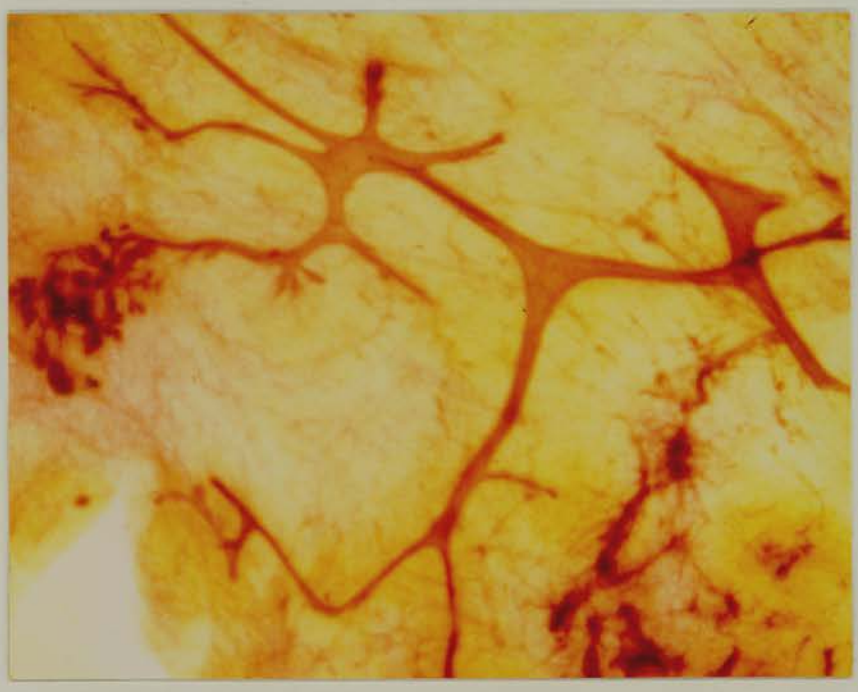
Field of cleared breast tissue (55 years). Dilations of the ducts may be observed at the junctions of more than three ducts.

Delafield's haematoxylin. (Mag. x 6).

Fig. 2.35

Field of cleared breast tissue (19 years). At the top right of the field, two stellate areas of duct branching are joined by a single duct of lesser calibre.

Delafield's haematoxylin. (Mag. x 6).



2·33

2·34

2·35

Fig. 2.36

Internal view of a wax model reconstructed from the left second thoracic gland of a newborn albino rat.

(Mag. x 40).

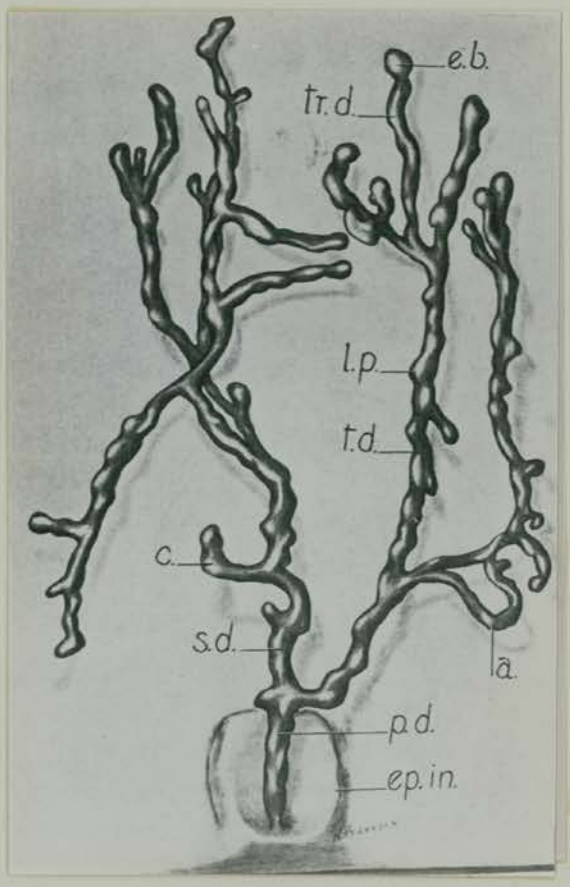
a - anastomosis
c - collateral duct
ep.in - epithelial ingrowth of nipple
e.b - end bud
l.p - lateral process
p.d - primary duct
s.d - secondary duct
t.d - tertiary duct
tr.d - terminal duct

Reproduced from Myers (1916).

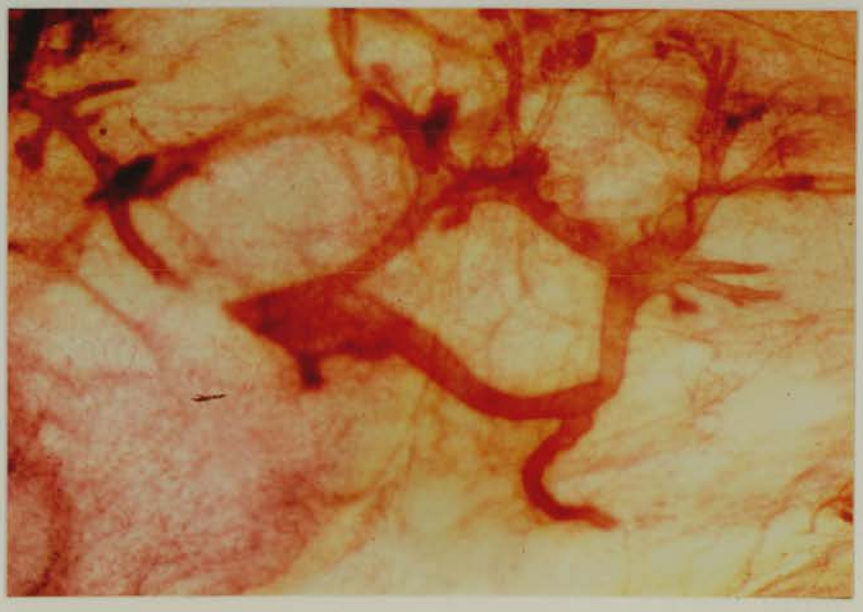
Fig. 2.37

Field of cleared breast tissue (35 years). In the centre of the picture, ducts appear to connect with each other to form a complete circle, an arrangement similar to the anastomosis described by Myers (1916) in Fig. 2.36. Serial sectioning to confirm this observation yielded equivocal results.

Delafield's haematoxylin. (Mag. x 18).



2·36



2·37

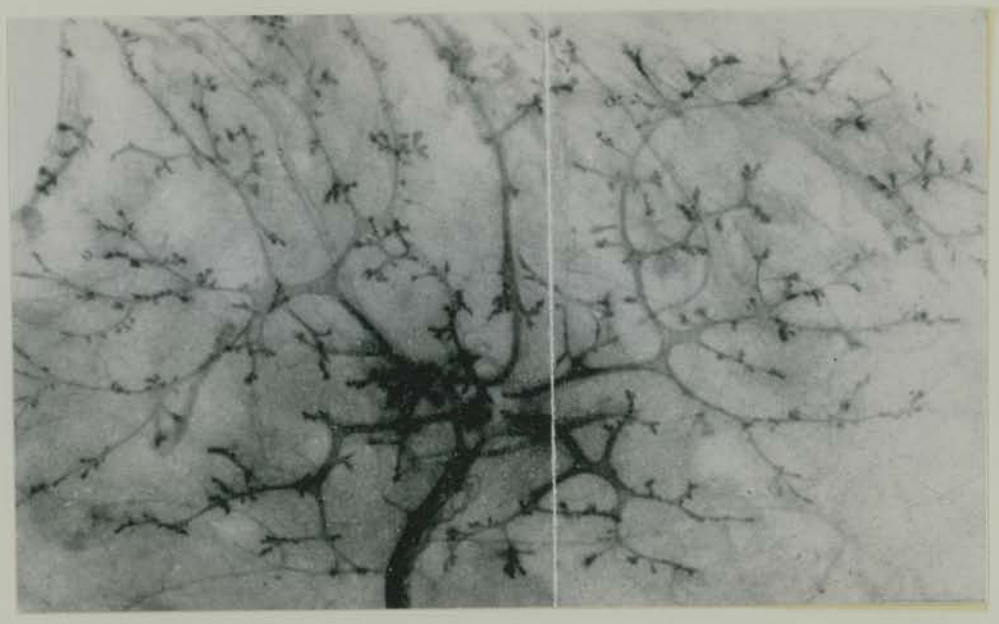
Fig. 2.38

Whole mount from a young rhesus monkey approaching puberty. From the series described by Van Wagenen & Folley (1939) and reproduced from Richardson (1947).

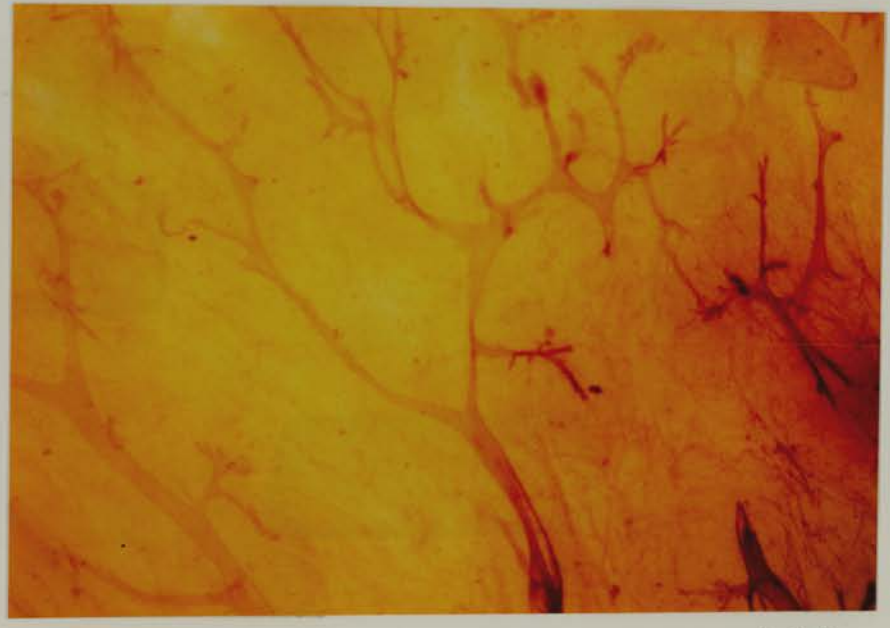
Fig. 2.39

Field of cleared breast tissue (46 years). The ducts branch in a manner closely resembling the branching patterns of the monkey mammary gland illustrated in Fig. 2.38.

Delafield's haematoxylin. (Mag. x 6).



2·38



2·39

Fig. 2.40

Whole mount of breast from mature IF virgin female mouse.

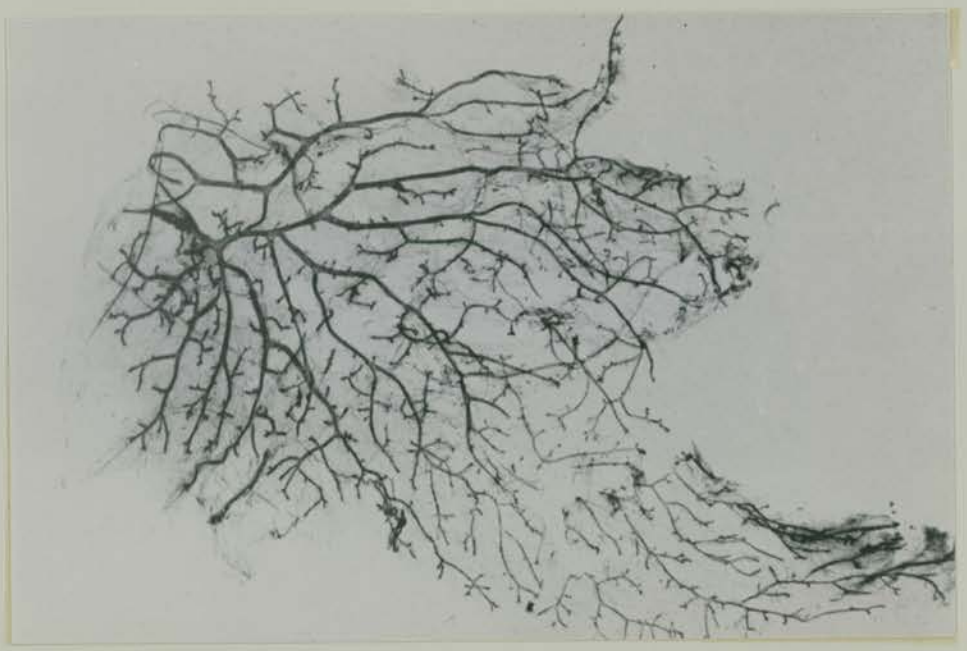
(Mag. x 5).

Reproduced from Bonser *et al.* (1961).

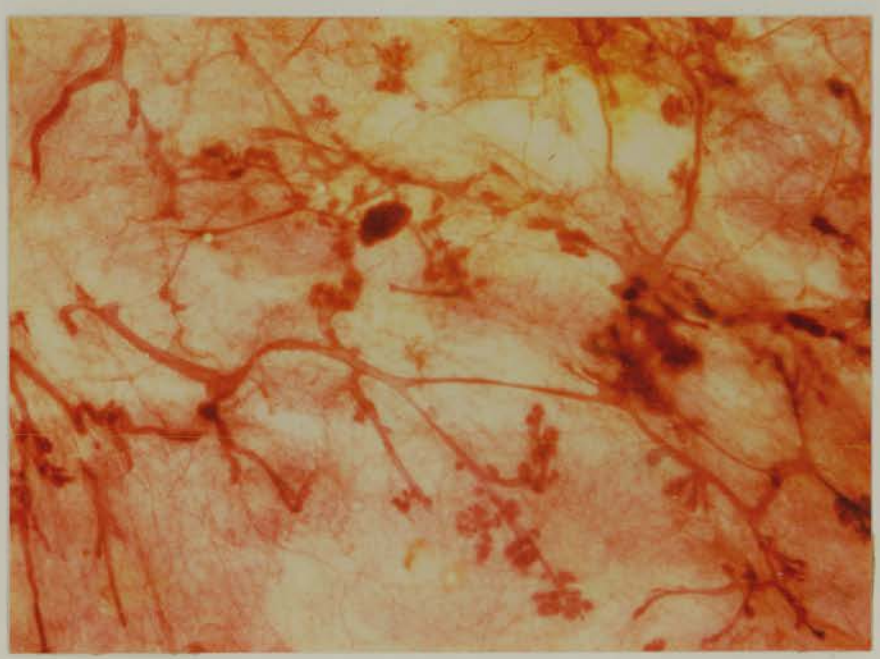
Fig. 2.41

Field of cleared breast tissue (38 years). The patterns of branching of the ducts bear resemblance to those in the mouse mammary gland illustrated in Fig. 2.40.

Delafield's haematoxylin. (Mag. x 6).



2.40



2.41

Fig. 2.42

Coronal slice of breast tissue - duct injection study (19 years). Mag. x 0.6.

a) Radiograph.

On the left of the picture, radio-opacities produced by the filling of ducts and parenchymatous lobules with micropaque may be observed.

b) Stained (Delafield's haematoxylin) and cleared tissue.

At the top left of the picture which is rotated relative to the radiograph, filling of the ducts and parenchymatous lobules with micropaque is seen as a black discolouration within these structures. This effect is more easily seen in the illustrations taken at higher magnification which follow.

2·42a

2·42b

Fig. 2.43

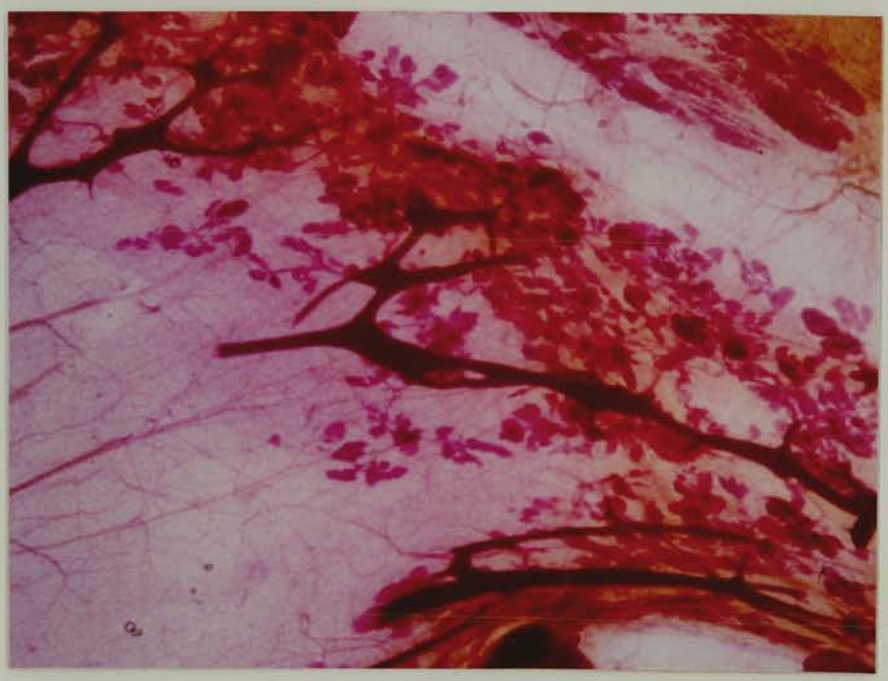
Field of cleared breast tissue (30 years) - duct injection study. The dark structures in the field from top left to bottom right are ducts filled with micropaque.

Delafield's haematoxylin. (Mag. x 6).

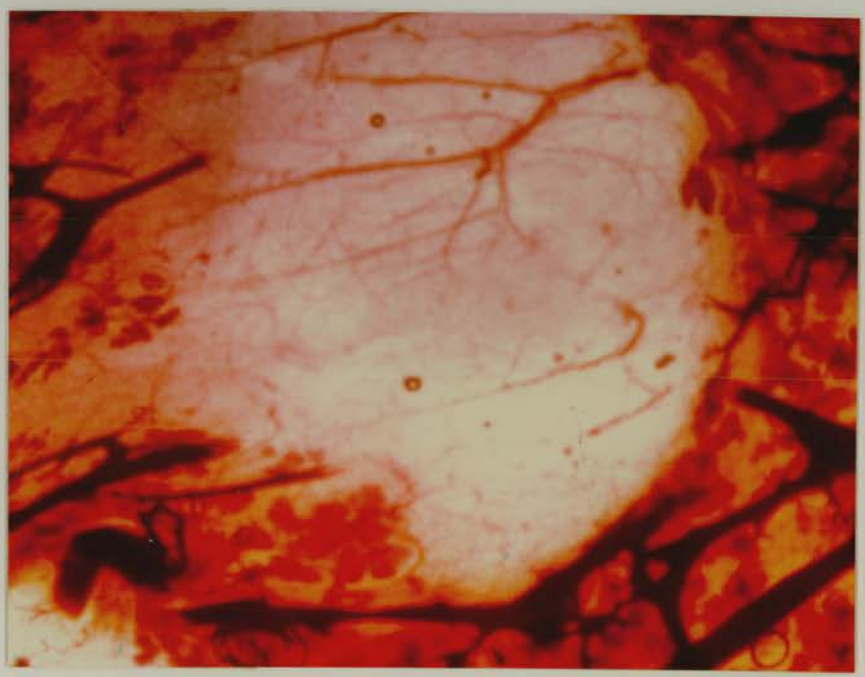
Fig. 2.44

Field of cleared breast tissue (30 years) - duct injection study. The darkly coloured ducts injected with micropaque may be seen ending abruptly at the periphery of a fat lobule and not travelling across it to connect at the other side.

Delafield's haematoxylin. (Mag. x 6).



2.43



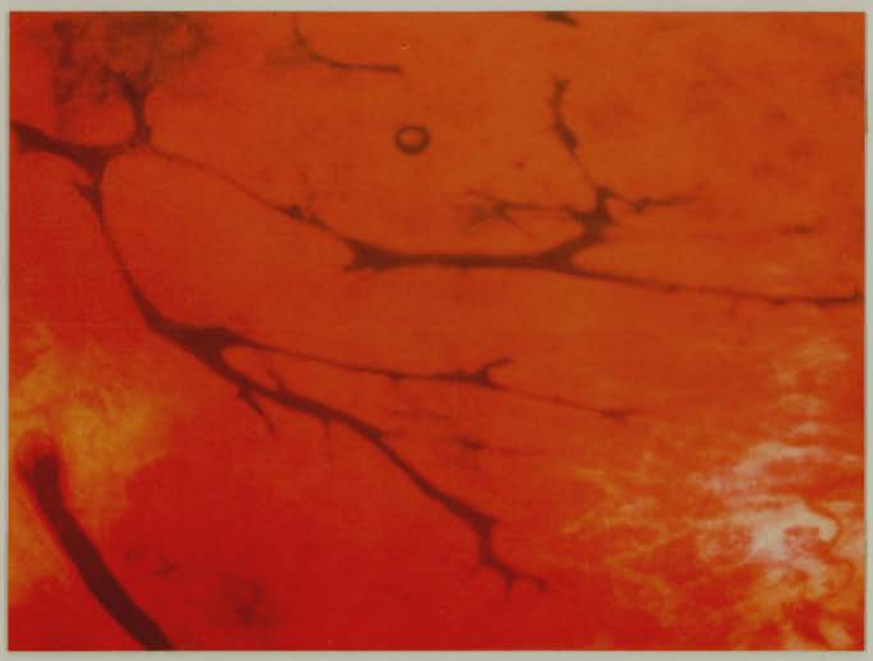
2.44

Fig. 2.45

Field of cleared breast tissue (40 years) - duct injection method. The dark structures travelling across the field are ducts which have been injected with micropaque. Although there are alterations in calibre of the ducts, supporting the observations made on ducts which have not been subjected to injection techniques, incomplete filling of the ducts with the injected material must not be excluded. Delafield's haematoxylin. (Mag. x 9).

Fig. 2.46

Field of cleared breast tissue (30 years) - duct injection study. In the centre and right of the field darkly pigmented ducts filled with micropaque may be seen terminating in parenchymatous lobules. Delafield's haematoxylin. (Mag. x 6).



2.45

2.46

Fig. 2.47.

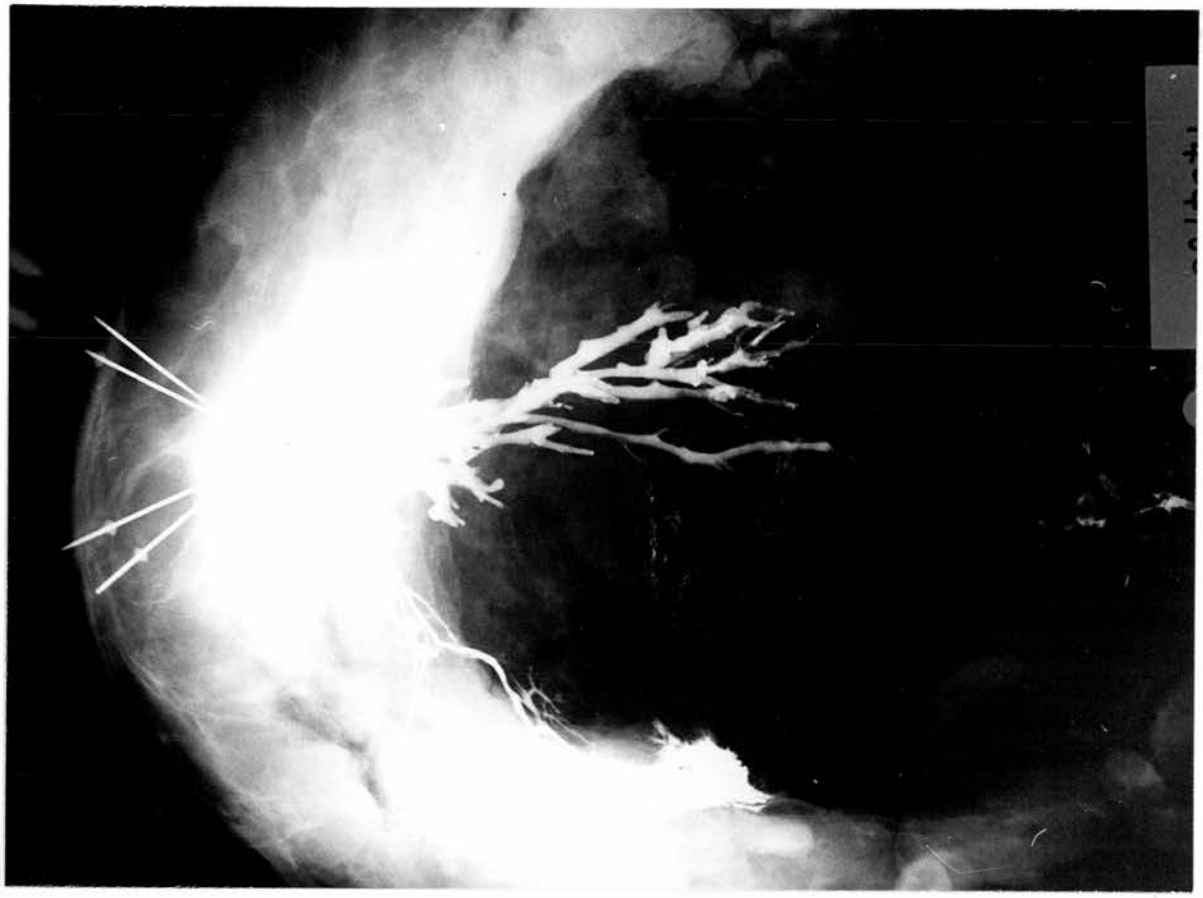
Radiographs of whole breast - duct injection study (54 years).

Mag. x 0.8.

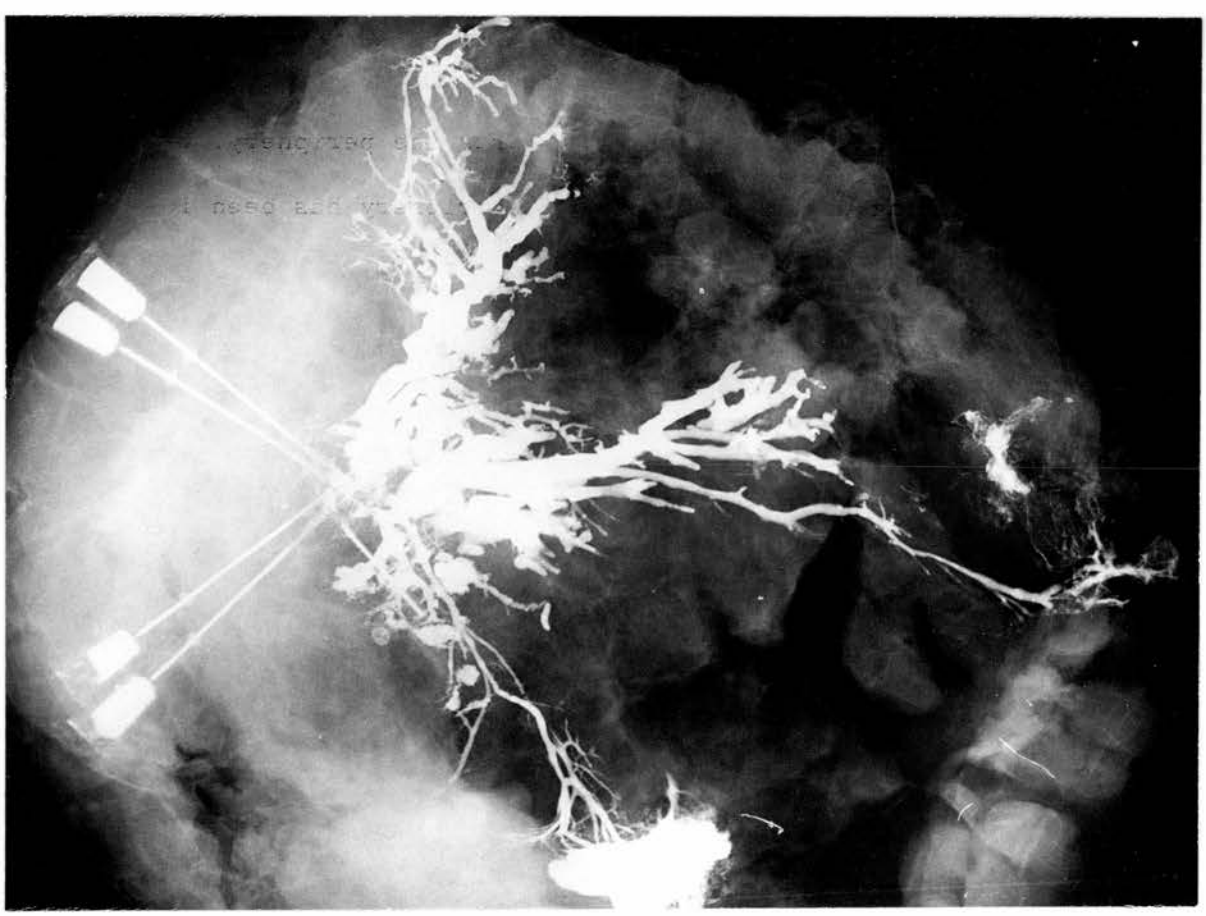
a) After injection of 3 ducts.

b) After injection of 5 ducts.

Ducts may be observed travelling horizontally to the right of the pictures. In photograph b) one lactiferous duct system overlaps another and extends further towards the periphery. The duct travelling furthest towards the periphery has been injected a second time to improve filling with micropaque.



2·47a



2·47b

Fig. 2.48

Radiographs of whole breast - duct injection study (29 years).

Mag. x 0.8.

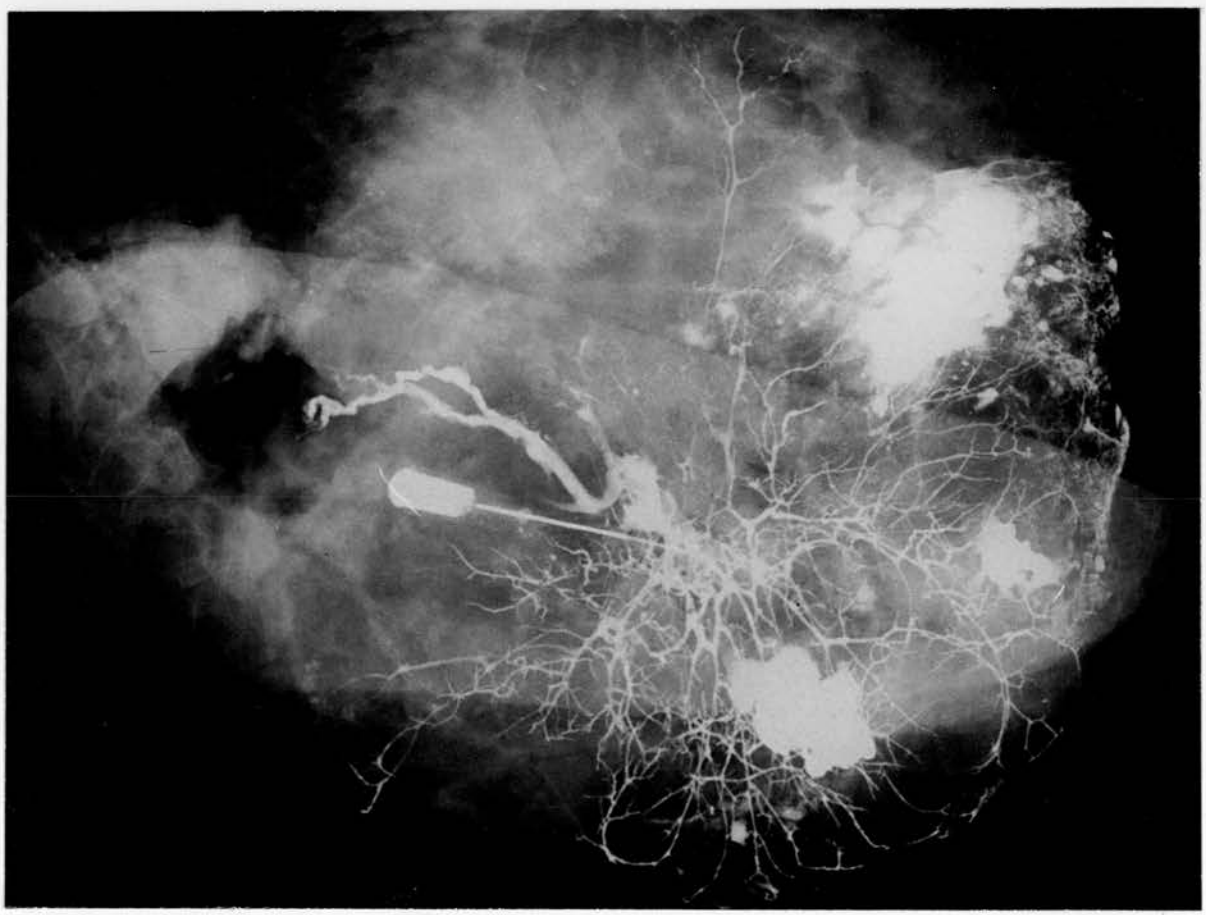
a) After injection of 3 ducts.

b) After injection of 6 ducts.

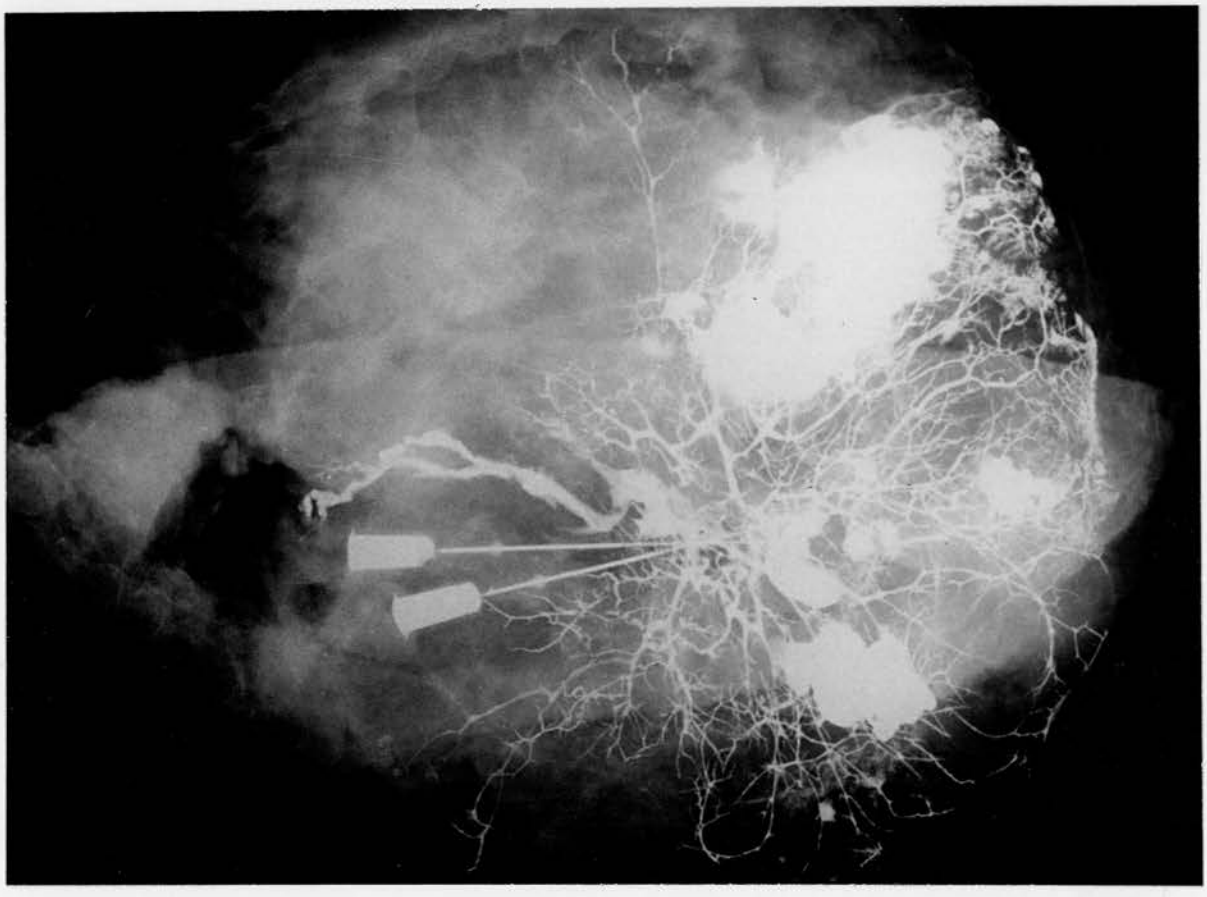
c) After injection of 7 ducts.

In each photograph the dense radio-opacities represent leakages of micropaque into the tissues; the pale radio-opacity running across the tissue is the skin ellipse; and the radiolucency to the left of the breast is a biopsy site. It can be observed that as the number of cannulations increases there is greater filling of the right side of the breast with injected ducts, which being intermingled do not provide evidence for a lobed structure to the breast.

2·48a



2·48b



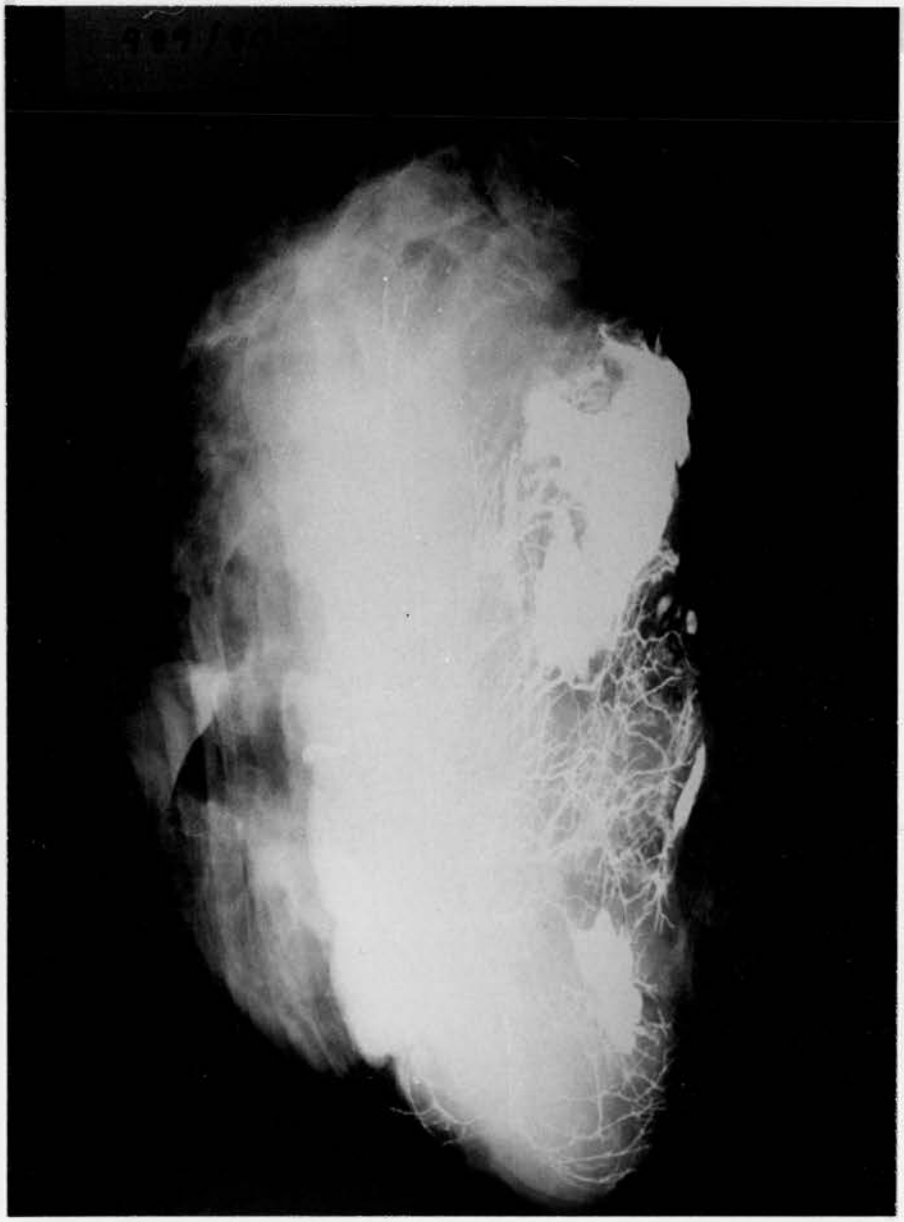
2·48c

Fig. 2.49

Radiograph of whole breast - duct injection study (29 years).

Mag. x 0.8.

The breast was radiographed at an angle of 45^{o} to the horizontal. At the bottom of the picture ducts may be seen travelling around the periphery of the breast. The dense radio-opacities are leakages of micropaque into the tissues.



2.49

Fig. 2.50

Radiograph of whole breast - duct injection study (29 years).

Mag. x 6.

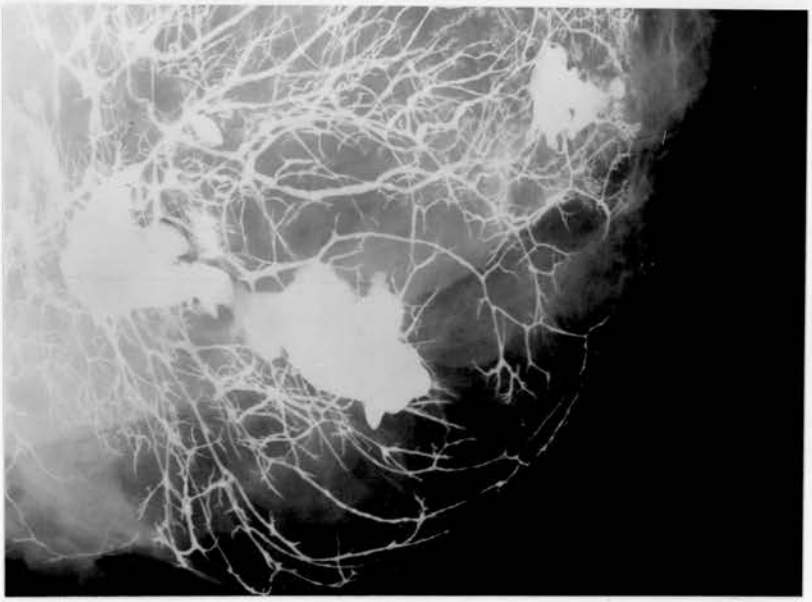
At the bottom of the picture ducts may be seen travelling along the periphery of the breast. The dense radio-opacities are due to leakage of micropaque into the tissues.

Fig. 2.51

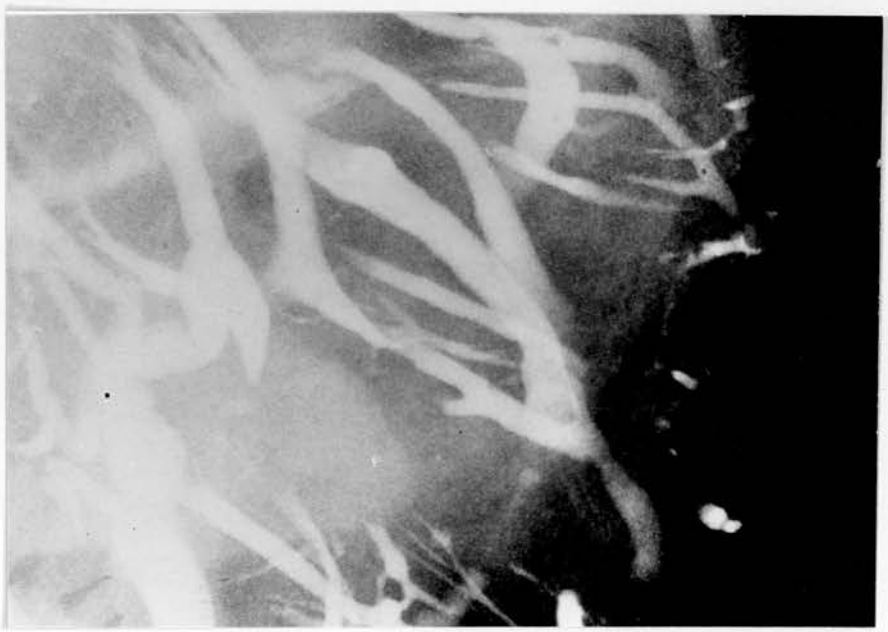
Radiograph of breast tissue - duct injection study (56 years).

Mag. x 6.

At the right of the picture ducts may be seen curving around the periphery of the breast.



2·50



2·51

Fig. 2.52

Radiograph of breast tissue - duct injection study (56 years).

Mag. x 6.

At the right of the field a duct may be seen turning back on itself to curve around the edge of the breast.

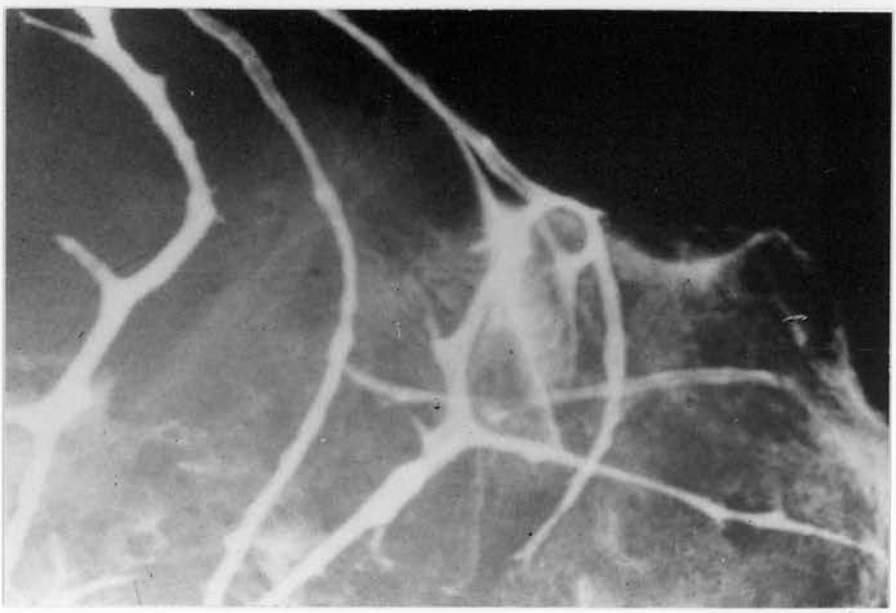
Fig. 2.53

Radiograph of breast tissue - duct injection study (29 years).

Mag. x 6.

In the centre of the field a duct loops around the periphery of the breast.

2·52



2·53

DISCUSSION

In this study the breast has been observed in a coronal, rather than a sagittal plane. This helps visualisation of parenchymal structures and, in particular, the passage of ducts through the tissues. Examination of the specimens revealed no evidence of the fibrous tissue septae which are said to partition lobes in the breast. If, for example, an orange was similarly sliced, the demarcations of the segments would be clearly visible. If the breast is divided into lobes, why cannot surgeons dissect out an affected lobe? There appears to be a discrepancy between theory and practice: surgeons realise that, other than biopsy, only "lumpectomy" or mastectomy are available as surgical procedures for the removal of lesions. There is no lobectomy, although this procedure might appear feasible on the basis of most current descriptions.

The fibrous connective tissue stroma and parenchyma were not always evenly distributed between the four quadrants of the breast, and considerable variation between breasts in the distribution and quantity of parenchymatous tissue was observed (Figs. 2.6-2.14). In very few specimens was the conventional pattern of duct branching observed with large ducts travelling from the nipple towards the periphery where the greatest concentration of lobules is expected. Lobules were commonly found occurring in considerable numbers in close proximity to the nipple (Fig. 2.15). In some breasts, no

major ducts were observed. This was largely due to numerous lobules obscuring the picture. However, as it is unlikely that all the major ducts in any one breast were cut at right angles to the plane of sectioning, this observation was somewhat surprising. Sizeable ducts were often seen ending abruptly at or very near the periphery of the cleared slices (Fig. 2.17). This observation may be explained by the fact that the breast "slumps" somewhat after removal from the body and the morphology of the duct system may therefore become distorted. On the other hand, autopsy breasts are quite firm and hold their shape well and therefore the observation may offer some support to Cooper's description of ducts forming a "hem" structure and doubling back at the edge of the breast.

Ducts travelling around the periphery of fat lobules were a common occurrence (Fig. 2.18) and both ducts and lobules were frequently observed along fibrous tissue septae surrounding fat spaces (Fig. 2.19). Whether these represent the ligamenta suspensoria of Cooper is difficult to ascertain especially with coronal sectioning. However, this feature might explain the high focal concentrations of lobules found occurring in the tissue slices and also why the parenchymal pattern may alter markedly from slice to slice. Cooper maintained that the ligamenta suspensoria formed loops and folds upon which lobules sat like the petals of a flower (Fig. 2.20). It is evident from observation of serial slices that parenchyma may pass down the sides of a fat lobule in the fibrous tissue until eventually a slice is reached in which the "floor" of a fat lobule is seen and there is a high concentration of parenchymal elements (Fig. 2.21). This phenomenon was found, as Cooper stated,

in the upper two-thirds of the breast. On occasion it was observed that the stroma appeared to be "stretching" the ducts and lobules and drawing them to follow its distribution (Fig. 2.22). The effect was one of a "whorled" type of pattern with the ducts travelling in a circular fashion, and it was difficult to decide whether the stroma or the parenchyma was dictating such a pattern. Individual ducts were also observed to follow sudden changes in direction, occasionally turning back on themselves (Figs. 2.23 & 2.24).

In the present study, stereomicroscopic examination of tissue slices and hand lens examination of black and white photographs was performed in an attempt to recognise and describe individual duct patterns. Ducts were seldom seen to branch regularly, i.e. in an isometric fashion, from the nipple to the periphery, but more frequently assumed an intermingling arrangement resembling a reticulum (Figs. 2.25 & 2.26). Indeed, it was on occasion very difficult to predict in which direction the ducts were "travelling", i.e. towards or away from the centre of the breast (Fig. 2.27) which would not be expected of true dichotomous branching. A regular arcading type of pattern quite often occurred in the slices and was not confined to any particular site in the breast (Figs. 2.28-2.30).

Adding to the impression that the ducts are arranged in an irregular branching network, it was observed that ducts displayed curious diameter changes and did not necessarily become smaller in calibre towards the periphery. Ducts of small diameter were seen "connecting" ducts of larger diameter (Figs. 2.26, 2.27, 2.30, 2.31,

2.32 & 2.35). This observation raised the question of whether the lactiferous duct systems are interlinked. Such a finding would not be outwith the normal anatomy of other parts of the body, e.g. the pores of Köhn in the lungs and capillary blood vessels. The diameter differences accentuate the dilations observed when more than two branches enter a duct (Figs. 2.33-2.35). Pathological change, in particular duct ectasia, could have contributed to these subgross appearances (Figs. 2.30, 2.31), notably in dilated ducts containing secretions. However, duct ectasia could not explain satisfactorily the phenomenon in the other illustrations. Similar dilations of breast ducts occur in both the monkey and the mouse breast (Folley, Gutkelch & Zuckerman, 1939; Huseby & Bittner, 1946).

It would be a difficult task to confirm that lactiferous duct systems in the breast connect with each other as it would be necessary to demonstrate complete continuity between one lactiferous system and another. However, the possibility that this arrangement exists need not be remote as connection has been demonstrated between ducts in the rat (Myers, 1916; Fig. 2.36). Also, sudden diameter changes resulting in a duct of small calibre joining ducts of larger calibre have been demonstrated in the submandibular salivary gland (Matthews, 1973). In this study, observation of Fig. 2.37 suggests that connection between ducts may be a real event.

Teleologically there would appear to be considerable biological advantage in some communication between duct systems allowing free drainage of blocked ducts; and, for maximum advantage, this must be peripheral. However, a simple explanation for the observation may

be that the connective tissue stroma and parenchyma are dynamic structures which, as a result of hormonal stimuli, constantly undergo alteration; and an uneven growth of one relative to the other may result in distortions affecting duct calibre.

Unfortunately attempts to trace the passage of individual ducts through the whole breast were unsuccessful and it did not prove possible to follow the ducts with any degree of accuracy from slice to slice of tissue. Attempts to apply quantitative methods to the arborisation of the ducts were also unsuccessful. The patterns in the human breast were too complex to apply methods used previously by workers on animal material who were able to observe the entire mammary gland in a single section using whole mount preparations (e.g. Gardner & Strong, 1935; Van Heuverswyn, Folley & Gardner, 1939; Cowie, 1947; Khanolkar & Ranadive, 1947; Silver, 1953a,b; Knobel, 1966).

Duct patterns observed in the human breast were compared to those reported in other animals. A striking similarity was found between some of the human specimens examined in this study and photographic whole mount reproductions showing the normal structure of monkey mammary glands (Gardner & Van Wagenen, 1938; Folley, Guthkelch & Zuckerman, 1939; Richardson, 1947; Figs. 2.38 & 2.39).

Similarities were also observed between duct patterns in the mouse and those in the human breast (Figs. 2.40 & 2.41). As in the human situation, very little work appears to have been devoted to defining duct patterns in the mouse. Khanolkar & Ranadive (1947) deal with duct patterns in their paper on the effect of foster nursing on the morphology of mammary glands in the mouse. Flux

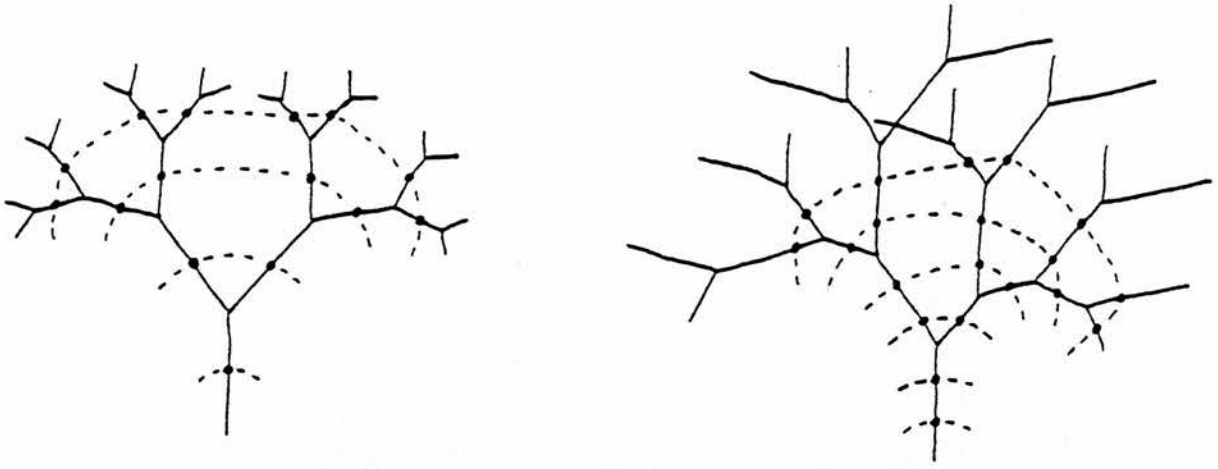
(1954a) estimated the degree of arborescence of ducts in the mouse and described isometric and allometric growth of ducts but neither individual nor recurring modes of branching. Knobel (1966), in his thesis on the development of the mammary gland in the mouse, made a detailed quantitative analysis of the number of end buds of the developing ducts, total duct length and the outline of the mammary gland area, but he did not describe the morphology of duct patterns. Other authors have concentrated on the effects on the parenchyma of hormonal stimulation (Turner, 1933; Gardner *et al.*, 1934; Gardner, 1935; Bonser, 1936; Fikete, 1938; Taylor & Waltman, 1940; Flux, 1954a,b; Ahrén & Jacobsohn, 1956; Bonser *et al.*, 1961; Evarts & Brown, 1977; Russo & Russo, 1978). However, their interest lay in finding which hormones produced growth of ducts, lobules, or both, in an attempt to throw light on the hormonal control and development of the human breast rather than in a description of duct patterns.

Branching patterns in the breast have been compared to those found in other biological fields (Thompson, 1942; Zimmerman & Brown, 1971; Stevens, 1976). If a network is to be distributed uniformly in space, then it must fan out at the periphery and a hierarchy of branches develops in which the small ones always outnumber the big ones. Horton (1945) analysed river systems and concluded that lungs, rivers and lightning have basically the same branching patterns based more on space than mechanism of flow.

Branching systems can be described in many ways, the most common of which is in terms of the 'order of branching'. For instance, a first order branch originating at a source receives no

other branches. A second order branch originates at the junction of two first order branches etc. Branches of higher order tend to be longer than branches of lower order and forks or junctions seldom exist at which more than three lines meet. Growth contours are lines which connect branches of either equal or unequal length (Fig. 2.54). The number on each contour indicates how many branches it connects. For example, if each branch divides equally into two, then the number of branches in a geometrical progression will be 1, 2, 4, 8, 16, etc. If long branches are split into two sections, on the assumption that they take twice as long to grow as short branches, then contours of equal growth connect branches according to the progression 1, 1, 2, 3, 5, 8, etc. where each term is the sum of the two preceding terms and is the name given to the Fibonacci series, which for a long time has been employed to describe patterns of branching (Richards & Schwabe, 1969; Williams, 1975; Dixon, 1981). The series is, however, only one of many number sequences that might be used to describe branching.

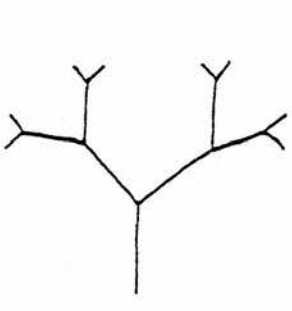
Murray (1926) investigated ways of describing the branching angles of arteries by applying principles of "least work". If a main artery is very large in comparison with a branch artery, blood will flow with less effort if it travels primarily in the large artery and minimises its journey in the small artery as near to 90^0 as possible. In other words, the smaller the branch, the closer to 90^0 will be the angle of divergence from the trunk. Trees also obey Murray's laws of least work (Murray, 1927), although the angles of branching are a little different from arteries. The branches of plants are found in two different positions: either opposite one



a) Contours connecting centres of branches of equal length

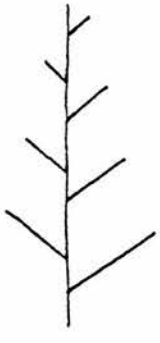
b) Contours connecting branches of uneven length

Fig. 2.54 EXAMPLES OF GROWTH CONTOURS

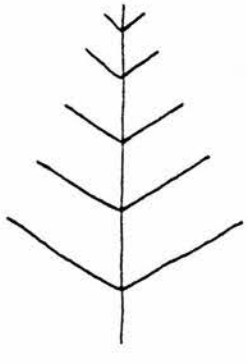


a) Dichotomous

b) Zigzag



c) Alternate



d) Whorled

Fig. 2.55 EXAMPLES OF PATTERNS OF BRANCHES IN TREES

another in which case the centre stem is not bent or not opposite, in which case the centre stem is bent. Branch angles vary in a narrow range of 75^0-90^0.

In trees the most primitive pattern of growth is dichotomous branching, in which one branch gives rise to two equal branches. If doubled branches grow unequally, then a zig-zag, i.e. overtopping pattern, develops. True lateral branches form an alternate arrangement and, with a third dimension, may grow in spiral succession around stems. A whorled pattern may also arise of branches which encircle the stem. Examples of these patterns are drawn in Fig. 2.55. Plants and trees put out stalks where they will have most room. The beauty and mathematics are by-products of a simple system of growth interacting with its spatial environment. Because of the environment, the pattern becomes modified. If it did not, the pattern would lead to conflict and leaves and branches would grow into one another. Therefore, success occurs because the pattern can be modified and, although Le Corbusier (1971) was correct in describing the tree as a mathematical function, it also responds directly to its environment.

Some trees exhibit a manifold habit of growth in which some branches are growing and others are not. It was observed by Koriba (1958) that, even in a uniform environment, many woody temperate plants develop the manifold habit. This is most noticeable in older trees where the positions of branches can create hormonal and nutritional differences. In the tropics, trees show cyclical periods of growth and rest (Alvim, 1964). Intermittent rather than continuous shoot growth is typical of most woody plants and it is difficult to explain why the majority of trees where

rainfall is even and temperatures constant conform to distinct patterns of cyclical growth. Roots will also show a periodicity of growth with inherent patterns of development which are modified by different environmental conditions, and will grow round an impenetrable object after which the root tip tends to re-orient to its original direction of growth.

It is evident that plants, trees and roots have complex growth patterns and that these inherited patterns alter with age under differing environmental and hormonal conditions giving rise to considerable individual variation. It is interesting that in some respects the breast may be described in terms of a tree but probably least of all for a simplistic dichotomous pattern of branching. Whorled, zig-zag and overtopping arrangements of ducts have all been observed in this study and the breast is also an organ which is reputed to alter its structures and to undergo cyclical changes throughout life. Attempts to apply recognised descriptions of branching systems such as the Fibonacci series to the breast material were unsuccessful. Only too often lobules obscured duct structures and, as stated, the patterns were not traceable from slice to slice of tissue. Radio-opaque injection techniques eased the task of tracing the ducts but using this technique to concentrate on a comprehensive study of duct branching in the breast was beyond practical possibility in this research. Ideally, material encompassing a wide age range and including very young individuals should be employed in an investigation attempting to throw light on the initial development of ducts and alterations occurring with age. Such material would be very difficult to obtain.

Examination of the radiographs of breasts following duct cannulations in both planes suggested that the duct systems are not arranged about the nipple with fibrous tissue septae partitioning lobes. Rather, they appear to have a layered arrangement with some ducts extending further towards the periphery than others. As the ducts divide in their passage through the breast tissue they become intermingled with the ducts and lobules of other systems and indeed resemble the roots of a tree as Cooper suggested (Figs. 2.47 & 2.48). Ducts were seen terminating abruptly at the edges of fat lobules (Figs. 2.43 & 2.44) and, at the periphery, ducts were observed to curve round the edge of the breast (Figs. 2.49-2.53) to form a kind of "hem". Confirmation of variations occurring in the calibre of ducts was also suggested (Fig. 2.46).

It was found that, in using the duct injection technique, the experiences in this study were in agreement with those of Hicken (1940) who reported that not infrequently injected ducts were found to come into intimate contact with the overlying skin. Because of this, injection material was occasionally found to leak onto the surface of the specimen.

SUMMARY

Selected aspects of the subgross architecture of the human breast have been described.

The breast does not appear to have a simple lobed structure. Rather, the 15-20 lactiferous ducts branch in such a manner that they intermingle and overlap each other and consequently cannot be individually dissected out. Some duct systems extend further towards the periphery and into the depth of the breast than others.

The ducts do not branch in a simple dichotomous fashion to terminate in lobules. Various morphological modes of branching may be observed and similarities exist between the human and both the monkey and mouse mammary glands.

Breast slices present varied appearances after application of staining and clearing techniques, depending on the ratio of fibrous connective tissue to fatty tissue and their distribution. The fibrous stroma and the parenchyma tend not to be distributed evenly between the four quadrants of the breast.

CHAPTER 3

QUANTITATION OF THE BREAST

INTRODUCTION

As stated in the general introduction, the majority of work on the human breast has been performed on pathological aspects of the organ and relatively little on the normal mamma. Of the work on the normal breast (e.g. Dawson, 1934, 1935a,b; Bonser *et al.*, 1961; Haagenson, 1971), very little deals with aspects of quantitation of the breast structures within the whole organ, particularly the non-fatty component consisting of parenchyma and fibrous connective tissue stroma (Engel, 1941; Sasano, Tateno & Stemmermann, 1978). Most accounts in the literature having a quantitative approach involve studies of whole organ volume changes during different periods of the menstrual cycle (Reimann & Seabold, 1933; Taylor, 1936; Milligan *et al.*, 1975) and deal with volumes of fluid increase and decrease within the breast. Many workers (e.g. Rosenburg, 1922, 1923; Polano, 1924; Foote & Stewart, 1945a; Ingleby & Gershon-Cohen, 1960) have discussed the possibility that the epithelial component of the breast grows and regresses with successive menstrual cycles; but this has been an opinion subjected to dispute and has not been expressed quantitatively.

In the literature, there are descriptions and classifications of breast types as well as data on individual breast shape and size. Such information is found in volumes on growth and anthropology (Montagu, 1960; Tanner, 1962), autopsy study (Sandison, 1962), and in work dealing with both pathology and normal structure of the

breast (Geschickter, 1945; Siemens, 1952). These classifications describe the breast in terms of gross appearance, size or firmness. They have been derived by measuring the ratio of fibrous stroma to fat using clinical palpation and whole organ sectioning. Such assessments have not been made on a quantitational basis. Sandison (1962) states: "I would point out the prevalence of the concept among surgeons and pathologists that the breast is an essentially fatty organ."

It is reported that there are different occurrences of tumour incidence in right and left breasts (Lane-Claypon, 1924; Busk & Clemmesen, 1947; Clemmesen, 1948; Harnett, 1948; Smithers *et al.*, 1952; Garfinkel, Craig & Seidman, 1959; Haagensen, 1971; Senie *et al.*, 1980) and also different occurrences of tumour incidence in the quadrants of the breast (Truscott, 1947; Harnett, 1948; Nicolson & Grady, 1948; Fitts & Donald, 1949; Deaton *et al.*, 1950; Smithers *et al.*, 1952; Tellem, Prive & Meranze, 1962; Donegan, 1967; Haagensen, 1971; Vorherr, 1974).

Busk & Clemmesen (1947) studied cases from the Danish Cancer Registry and found that, for each 100 cases of cancer in the right breast, there were 111 in the left breast. Nearly 30 years later, Haagensen (1971) reported that his findings also agreed with these figures. Amongst other workers who have concluded that left-sided cancers are more frequent than right are: Lane-Claypon (1928), Harnett (1948) and Smithers *et al.* (1952). Senie *et al.* (1980), in a study of 980 patients, revealed a left/right ratio of 1.26 and disclosed a significant association between left predominance and

clinicopathologic features which included menarche after the age of 13 and parity. They computed breast volumes from the mammograms of 174 healthy women and found that 55% had a larger left breast. They suggested that the asymmetry of breast carcinoma reflects differences in the sensitivity of the mammary glands to hormonal stimuli resulting in unequal volumes of tissue at risk to developing carcinoma. However, the mammary glands of breast cancer patients were not found to be considerably larger when compared to controls in studies of British (Katariya, Forrest & Gravelle, 1974); American (Wynder, Bross & Hirayama, 1960; Hirohata, Nomura & Kolonel, 1977) or Finnish (Soini, 1977) women. A review of the literature on the occurrence of right- and left-sided cancer was performed by Garfinkel *et al*. (1959) who found that cancer was indeed more frequent in left than right breasts and stated that:

> one possible area of investigation to explain the left/ right difference concerns the physical size of the left breast as compared to the right. It is conceivable that the greater occurrence of left breast cancer is attributable to the fact that, in the general population, there is more breast tissue in left than right breasts. It would admittedly be difficult to determine how the breasts should be measured, but even a small scale study designed to test this hypothesis would be interesting.

On considering the frequency of tumour incidence in the different quadrants of the breast, Smithers *et al*. (1952) reported more tumours occurring in the upper outer quadrant "because this quadrant contains more volume of breast tissue". They comment that the uneven site distribution of primary breast cancers has not been satisfactorily explained but may be due to no more than the relative amounts of breast tissue present. However, according to Newton (1961) "the problem of measuring the amount of glandular

tissue in a given breast in the living woman is virtually impossible to solve."

Haagensen (1971) reviewed several case series of breast cancer (e.g. Truscott, 1947; Harnett, 1948; Smithers *et al.*, 1952; Donegan, 1967), which included data as to the site of the tumour in the classical four quadrants as well as the central region of the breast (defined as an area having a radius of 1 cm around the nipple). Overall the series showed the highest tumour incidence to be in the UOQ ranging from a 30.6% to a 48.0% occurrence. The quadrant with the next highest figures was the UIQ followed by the LOQ and then the LIQ. Tumour incidence in the centre of the breast exceeded that in all the quadrants excepting the UOQ. Haagensen (1971) makes the point that the data in the case series that he reviewed were based upon written descriptions of the site of the tumour and that they are therefore not as precise as data based upon actual sketches of the breast, or upon diagnosis of the breast in which the various breast sectors are specified. In his clinics Haagensen used a diagram for recording the clinical findings in mammary carcinoma, in which the mammary gland was divided into seven carefully delineated areas. He found in his own data (a series of 1007 patients) that, as in those from other sources, the most frequent site of carcinoma is in the UOQ with a percentage incidence of 38.5%, followed by the UIQ, 14.2%, LOQ, 2.8%, LIQ, 5.0%, and with central lesions accounting for 29% of tumour occurrence. Haagensen states that, in the UOQ:

> It may be argued that this sector contains a greater bulk of breast tissue than any of the other three quadrants; that is, it contains the greatest number of cubic

> centimetres of mammary gland exposed to the risk of carcinomatous change. This is a purely anatomic explanation, but I know of no other.

A small proportion of modern surgical texts including Rush (1974), Wilson, J.L. (1981) and Wilson, R.E. (1981), also state that more tumours occur in the UOQ of the breast, all three texts indicating over 40% occurrence. Only Wilson, J.L. (1981) makes a comment on this figure: "Almost half of cancers of the breast begin in the UOQ probably because this quadrant contains the largest volume of breast tissue." The same text, reporting that cancer occurs 5-10% more frequently in left than right breasts, suggests that there is "no satisfactory reason for this".

Lane-Claypon (1926), in a paper on cancer of the breast with special reference to its associated antecedent conditions, attributed the predominance of left-sided breast cancer to injury "presumably" because most people are right-handed and protection of the right side is more efficient. In her material, a higher proportion of left breast injuries were observed and there was correlation between cancer incidence and antecedent injury. However, the difficulties surrounding establishing a case of single trauma breast cancer are outlined by Stevens (1978). It is the opinion of Tashima (1974) and Senie *et al.* (1980) that injury just prior to diagnosis draws attention to pre-existing lesions that have been latent and is not a causative aspect of breast cancer. Salk (1970) reports that women more often carry their infants on the left side and speculates as to whether this frequent although not severe trauma is related to cancer incidence.

Deaton *et al.* (1950), in a paper entitled, "Carcinoma of the breast - why the upper outer quadrant?" stated that aberrant breast tissue is frequently found in the UOQ (Hertzler, 1933) and suggested that this aberrant tissue has a special tendency towards tumour formation. Quoting Cummins (1943) as their reference that supernumerary mammae and nipples are more frequent on the left rather than the right side, they suggested that this could explain the left-sided predominance of tumours. However, perhaps the most bizarre explanation for the "vast majority" of tumours occurring in the UOQ was put forward by Nicolson & Grady (1948). They maintained that there are more tumours and pre-existing mastitis in the UOQ because the breast is not properly supported when the woman is upright, and concluded that a well-designed brassière would do a great deal towards preventing carcinoma.

There is clearly a lack of information regarding the distribution of the parenchyma and the fibrous connective tissue stroma within the normal breast. The literature suggests a need for some form of quantitative analysis and, by using a quantitative approach, several aspects of the normal organ may be considered.

The research described in this chapter was performed in two stages: a preliminary investigation followed by a main study. The preliminary work will be discussed first and involves a detailed quantitation of the lobules as well as the non-fatty breast tissue component (NFC) in a small series of 10 whole breasts. Following the conclusions of the preliminary study, procedures were modified so as to allow a further 20 specimens to be quantified. The studies are concerned with the distribution of lobules and the NFC

within the breast, in particular the variation between quadrants and between the superficial, middle and deep thirds of the organ. Moreover, both lobules and the NFC are related to age, parity, laterality, tumour incidence and comparison of paired specimens.

What is defined in this study as the non-fatty component (NFC) of the breast is recognised as fibrous connective tissue when the breast is sectioned. It is composed of extralobular and intralobular stroma, and the parenchymal elements which are ducts and lobules. It is not possible to separate the parenchymal and stromal elements in slices of breast tissue without staining and clearing procedures. Therefore, visual and radiographic quantitation of unstained tissue necessarily includes parenchyma in the stromal portion.

PRELIMINARY STUDY

MATERIALS

Whole breasts were obtained from autopsy suites and operating theatres. The majority of female subjects at autopsy are post-menopausal, and most premenopausal breasts had to be obtained from surgical excisions (mastectomies).

In the preliminary study, 10 breasts were examined and the details of these are listed below:

Age (in years)		Quadrant of tumour involvement
Right breasts		
*19	autopsy	-
19		-
22		-
50		-
*63	surgical mastectomy	-
Left breasts		
*19	autopsy	-
26	surgical mastectomy	LOQ
43		UOQ
43		UOQ
*63		

*Matching pairs of right and left breasts.

METHODS

Processing of the whole breast specimens was performed as described in chapter 2. Each specimen in the preliminary study was

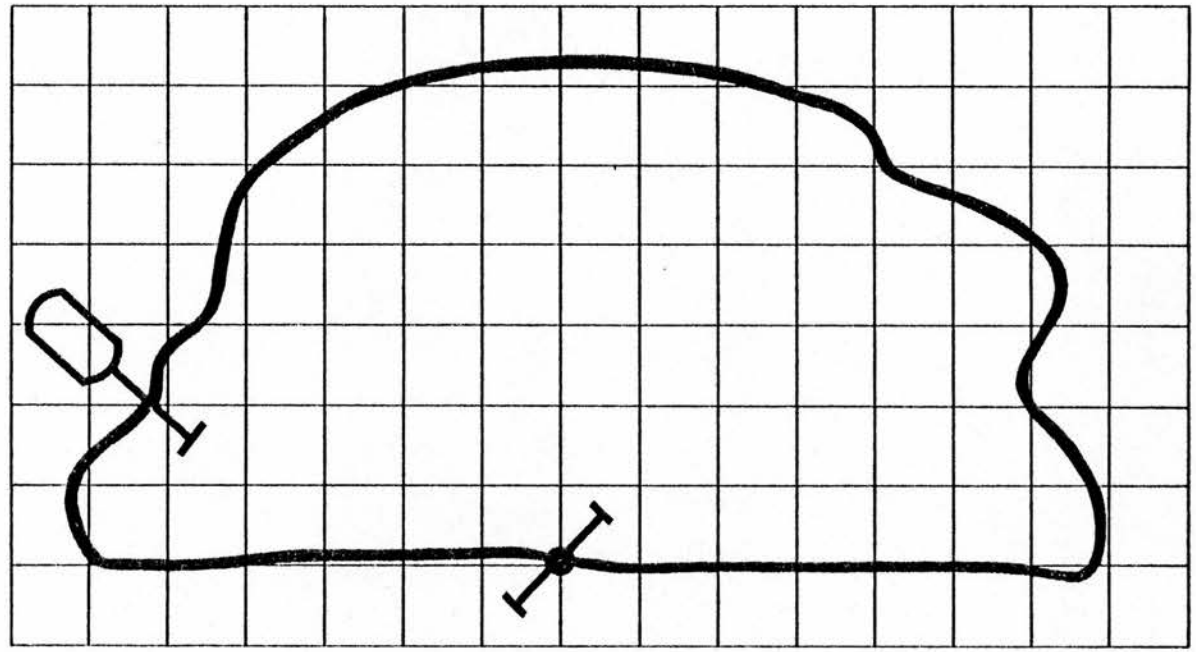
sliced in two and each half sectioned coronally.

Quantitation of Lobules

The method of processing resulted in slices of breast tissue in which the epithelial component, comprising ducts and lobules, was stained and the background connective tissue, consisting of fat and fibrous tissue, was cleared. The 2 mm thickness of the slices allowed visualisation of the stained parenchyma throughout each entire slice.

To quantify the number of lobules, an acetate grid divided into 1 cm^2 cells was placed underneath a 30 x 20 x 3 cm glass pot. The pot contained a slice of tissue submerged in methyl salicylate. The slice was secured in position beneath a glass plate and was so placed that the straight edge of the slice lay as nearly as possible along a horizontal line of the acetate grid. The centre of the slice, marked by a polythene tag, coincided with a vertical line on the grid. In this manner the slice could be divided into upper and lower halves and, if required, could be re-orientated with ease for further examination (Fig. 3.1).

For the purposes of this research a lobule was defined as "a circumscribed collection of ductules with or without visible attachment to a terminal duct". Counting of lobules was performed using a Wild-Heerbrugg (Leitz Ltd., Luton, England) M8 stereo-microscope at x 9 magnification. Lobules covering the lines of the acetate grid were included in the count for the cell over which most of the lobule was contained. Lobule numbers were assessed in the



identification tag marking upper portion of the breast

identification tag at nipple position corresponding with a vertical line on the $1cm^2$ acetate grid

Fig. 3.1 Diagram to illustrate orientation of coronal slice of breast tissue bisected in a sagittal plane over the $1cm^2$ acetate grid used for quantitation of the parenchymatous lobules.

following categories:

Category	No. of Lobules
0	0
1	1- 10
2	11- 20
3	21- 30
4	31- 40
5	41- 50
6	51- 60
7	61- 70
8	71- 80
9	80- 90
10	91-100
11	101-125
12	126-150
13	151-200
14	201-250

The lower the concentration of lobules, the easier was assessment and counting. For the first 10 categories, i.e. up to 100 lobules, assessment to within 10 lobules was possible. For the higher categories (11-14), the density of lobules was such that accuracy to within 10 was impossible as distinction between individual lobules became progressively more difficult. Hence, the two highest categories comprised 50 lobules each. In spite of a difficulty encountered in separating lobules in areas of greatest concentration, the assessment of the highest category was estimated as being comfortably within the upper limit of 250 lobules/cm^2. The lobules present in all of the slices in each of the 10 breasts were counted.

Quantitation of the Non-Fatty Component (NFC)

At three stages during the processing of the specimens hand tracings were made of all the slices in each of the 10 breasts studied:

1. on completion of embedding, slicing and fixation in formaldehyde.
2. On completion of staining and dehydration of the slices.
3. After clearing the tissue slices in methyl salicylate.

The slices of breast tissue were placed on a flat surface with a covering glass plate. A fine tip felt pen with permanent ink was used to trace the margin of each whole slice as well as the outline of the NFC within it onto the glass. After the slices had been cleared in methyl salicylate, only the periphery of the slices was recorded as the outline of the NFC had been obliterated by the clearing process. The tracings on the glass were then transferred to tracing paper.

A semi-automatic, computer-assisted image analyser (GDSI 4051, Tektronix UK Ltd., Herts., England) was used to record from the tracings the total area of each slice of breast tissue and the area of the NFC in mm^2. The area of fat in each slice was calculated by subtracting the area of the NFC from the total area. All of the slices in each of the 10 breasts were measured. Readings were converted from mm^2 to cm^2 for presentation of the results.

RESULTS

Quantitation of Lobules

The number of lobules was calculated from the recorded categories as follows: the figure at the midpoint of each category was taken as the number of lobules representing that category. The midpoint values are listed below:

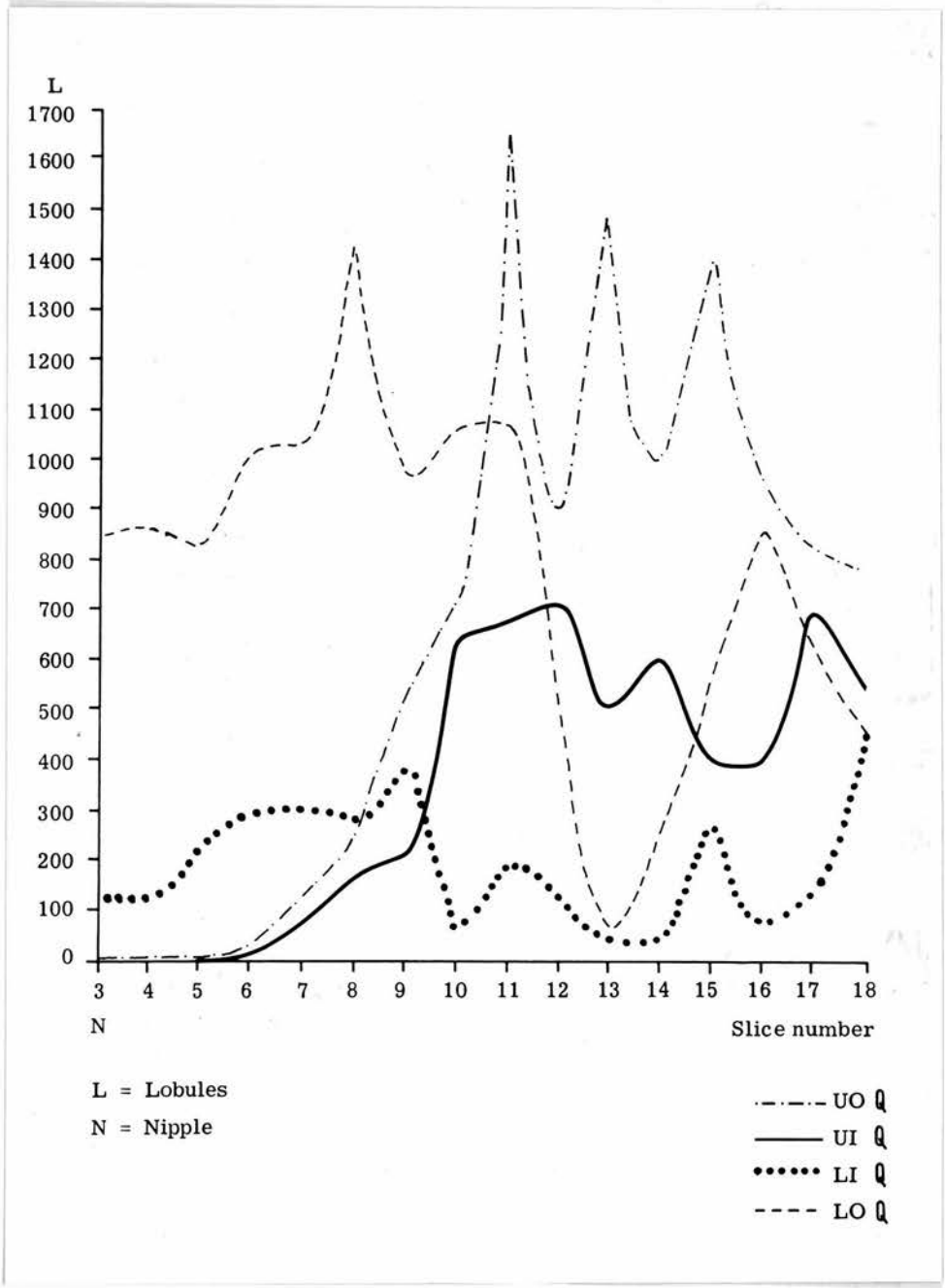
Category	No. of Lobules
0	0
1	5
2	15
3	25
4	35
5	45
6	55
7	65
8	75
9	85
10	95
11	112
12	138
13	175
14	225

The number of times the various categories were recorded in each half of a slice of tissue was multiplied by the appropriate midpoint values to produce a figure for lobule number. The number of lobules could subsequently be expressed in terms of individual slices, quadrants and the whole breast.

The number and distribution of lobules in the series of 10 whole human breasts may be consulted in Appendix C, Table 4. This shows that total lobule numbers varied with age. Confirmation of this finding was obtained by calculating the Spearman's rank correlation coefficient for the sample, which showed that lobule

Fig. 3.2

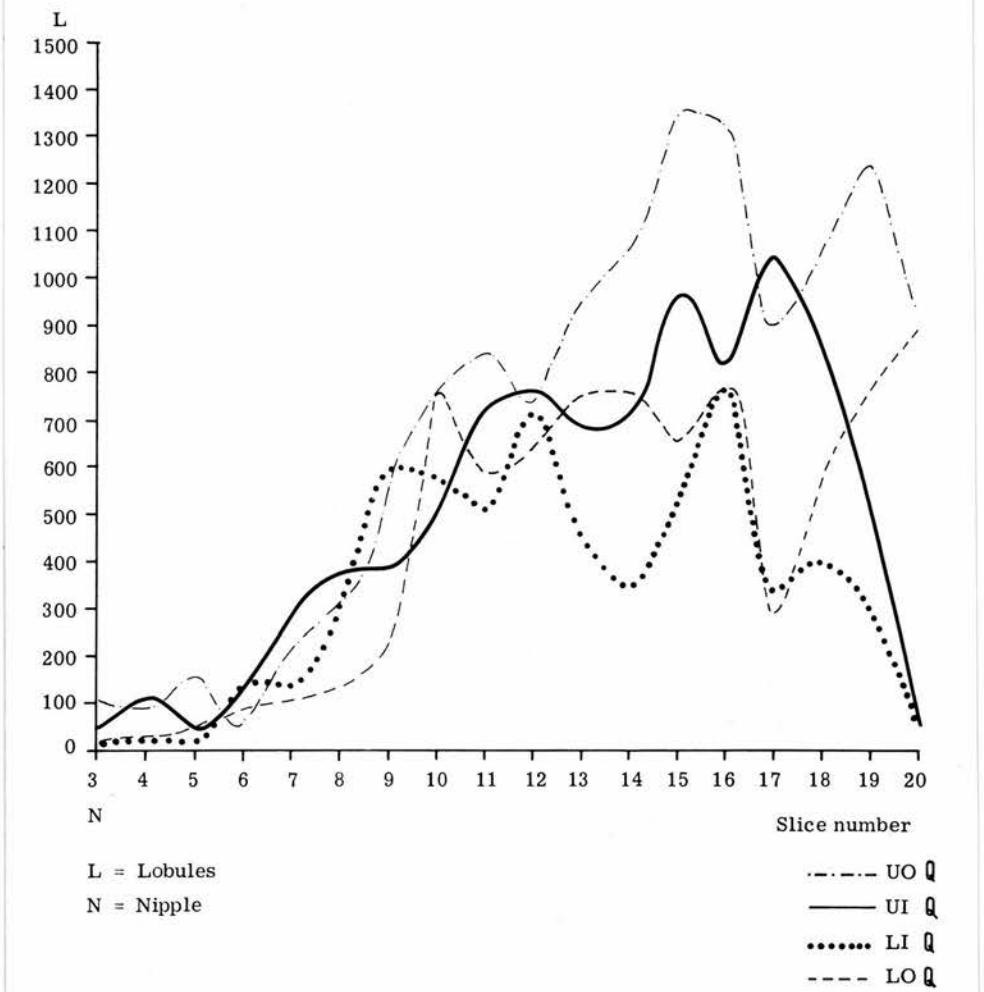
Number of lobules per slice of breast tissue plotted against slice number for each quadrant of the breast. The specimen was the left breast from a 26 year old woman.


L
1700
1600
1500
1400
1300
1200
1100
1000
900
800
700
600
500
400
300
200
100
0
3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
N
Slice number
L = Lobules
N = Nipple
UO Q
UI Q
LI Q
LO Q

3·2

Fig. 3.3

Number of lobules per slice of breast tissue plotted against slice number for each quadrant of the breast. The specimen was the left breast from a 43 year old woman.


L
1500
1400
1300
1200
1100
1000
900
800
700
600
500
400
300
200
100
0
3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
N
Slice number
L = Lobules
N = Nipple
UO Q
UI Q
LI Q
LO Q

3·3

numbers significantly decreased with age ($p < 0.02$). Table 4 also illustrates the percentage of the total number of lobules found in all the quadrants of the breasts. This shows considerable variation between the four quadrants in any one breast. There is also variation between the same quadrant from different breasts.

In only one of the right breasts and four of the left breasts did most lobules occur in the UOQ. Of the four specimens which were surgical mastectomies, the quadrant containing the tumour site also contained the greatest number of lobules. It was noted that the largest breasts did not necessarily contain most lobules. Further, women of 50 and 43 years had similar lobule numbers as well as density of lobules per cm^2 of tissue to women of 22 and 26 years.

The number of lobules per slice of breast tissue was plotted against slice number for each quadrant of the 10 breasts examined. Figs. 3.2 and 3.3 are examples of the plots which were obtained from the left breasts of two women of 26 and 43 years of age respectively. The distribution of lobules in the four quadrants is shown. Fig. 3.2 illustrates a marked difference in lobule content between quadrants, whereas in Fig. 3.3 this difference is not so marked. Both graphs are peaked showing the slice to slice variation that occurred in lobule number. It can be seen that lobule numbers increased from the nipple towards the base of the breast. Slices nearest the base of the breast however did not display the greatest lobule content.

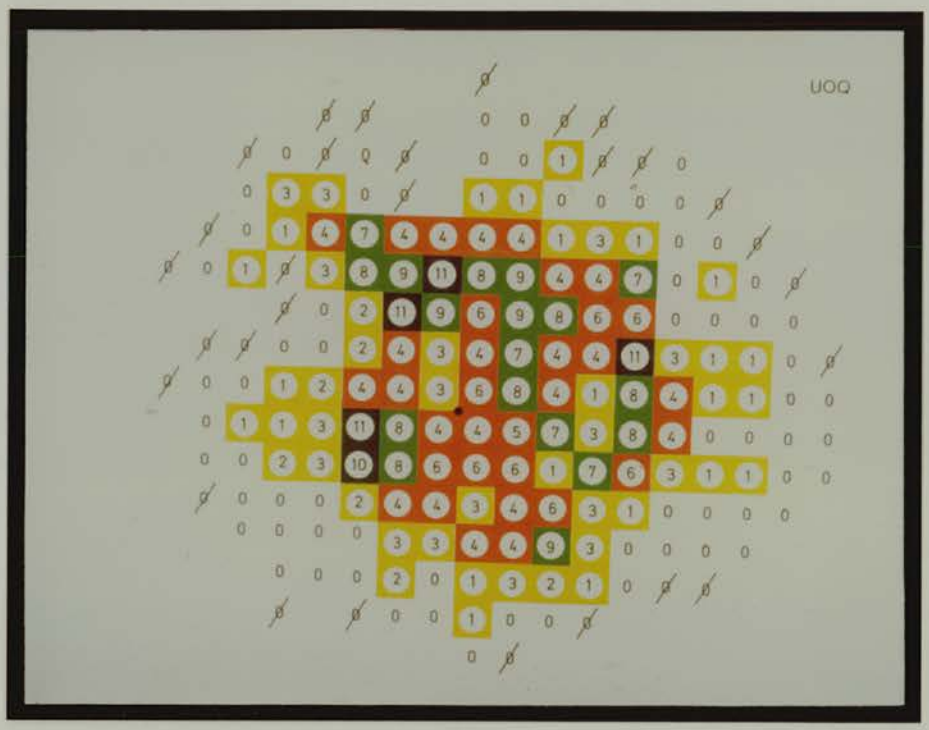
Breast "maps" were constructed by superimposing the results for lobule counts upon a diagram of the cm^2 grid to examine the changes

Fig. 3.4

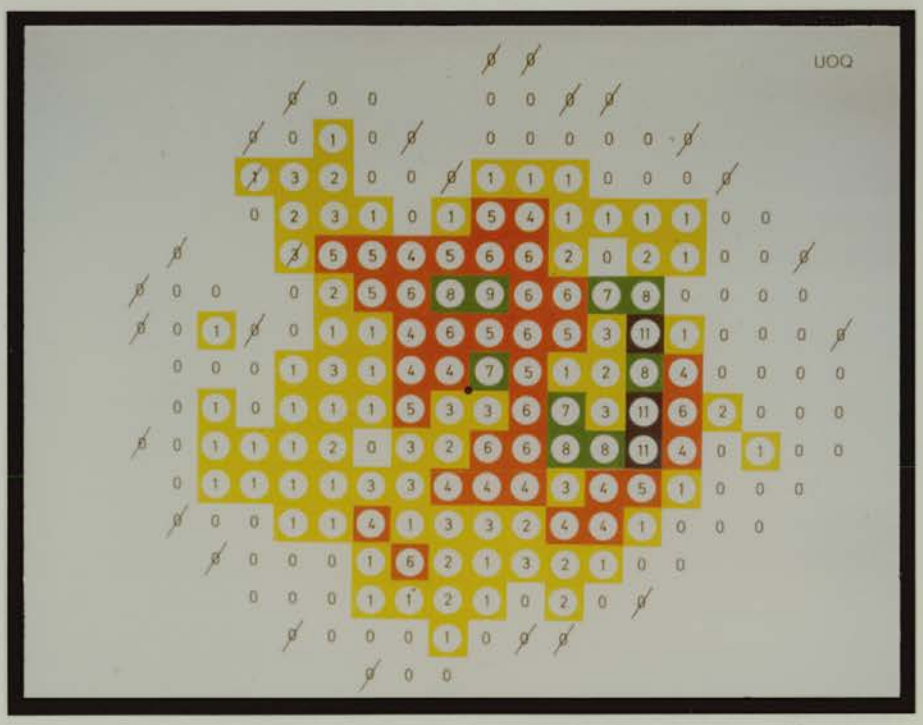
Breast maps of two adjacent coronal slices of breast tissue illustrating foci of varying parenchymatous lobule concentration from slice to slice of tissue (43 years).

Lobule categories:

1 - 3	=	yellow
4 - 6	=	red
7 - 9	=	green
10-11	=	black



3·4a



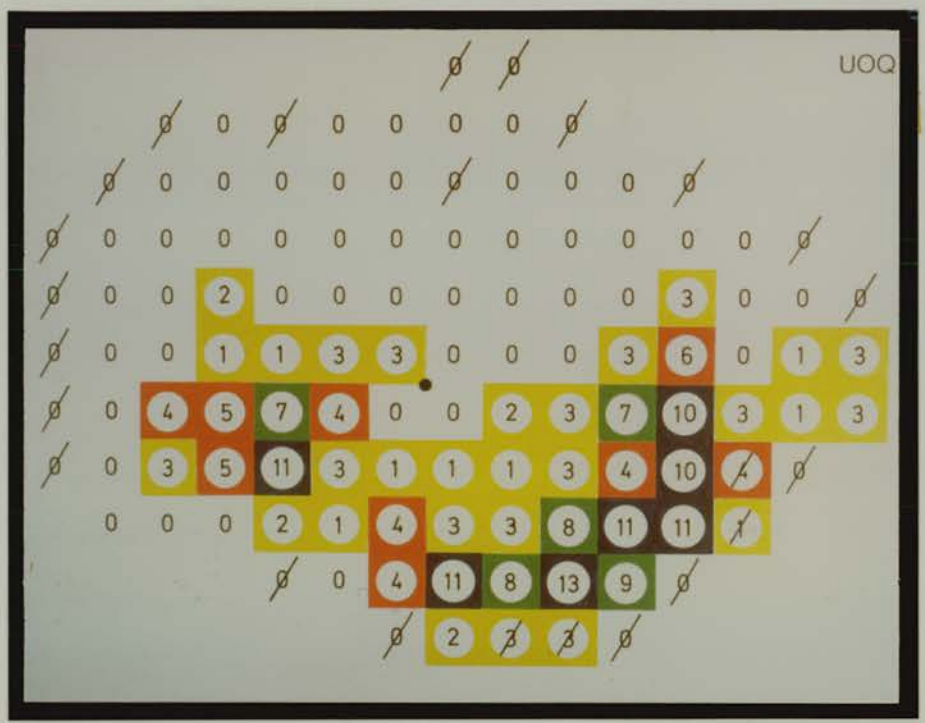
3·4b

Fig. 3.5

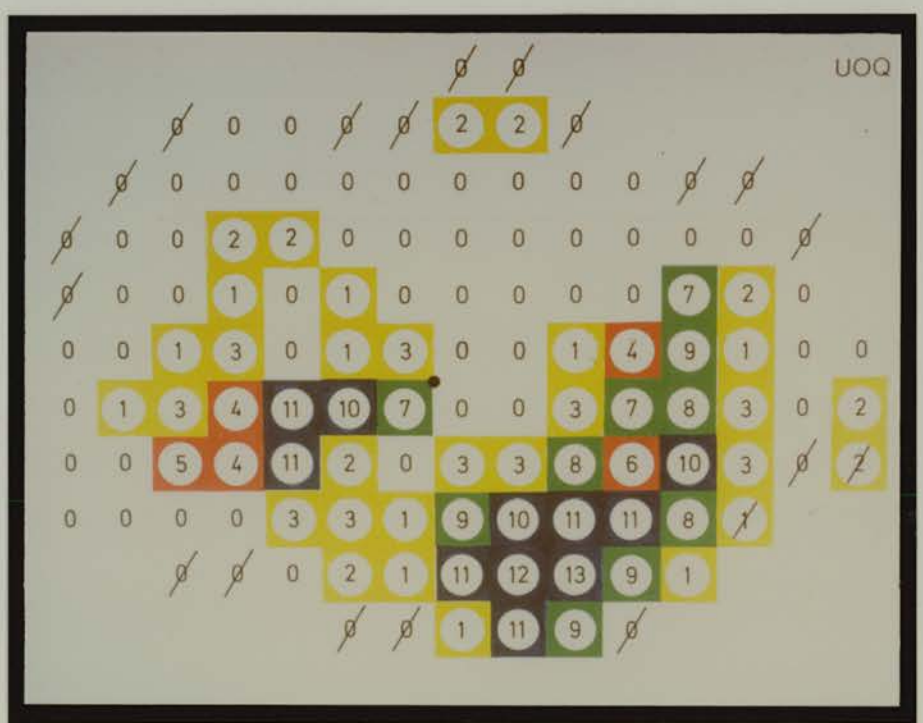
Breast maps of two adjacent coronal slices of breast tissue illustrating an uneven distribution of parenchymatous lobules between quadrants (26 years).

Lobule categories:

1 - 3	=	yellow
4 - 6	=	red
7 - 9	=	green
10-13	=	black



3·5a



3·5b

in the concentration of lobules from slice to slice of tissue, and also the distribution of lobules between quadrants. Examples are provided in Figs. 3.4 and 3.5, which illustrate respectively foci of high concentrations of lobules occurring within the breast, and uneven distribution of lobules occurring between quadrants.

Quantitation of the Non-Fatty Component

The results for the 10 breasts which were traced and measured are presented in Appendix C, Tables 5-7.

The figures obtained for the total area of breast tissue recorded in each quadrant of the breast after fixation and after staining are presented in Table 5 which also includes the number of lobules contained in each quadrant. It can be seen that the largest quadrant does not necessarily contain the greatest number of lobules.

The area of the NFC recorded in the quadrants of the 10 breast specimens after fixation and after staining is shown in Table 6. The considerable discrepancy between the values for the two sets of data should be noticed.

Table 7 illustrates the percentage of the NFC and fat comprising the total area of breast tissue in the 10 breasts after fixation and after staining. As in Table 6, the discrepancy between the data recorded for the sets of measurements should be noted.

DISCUSSION

Quantitation of Lobules

As a result of the preliminary study, difficulties in the technique for quantitation became apparent. First, and most important, it could be difficult to distinguish accurately one lobule from another. The edges of some lobules were very well circumscribed and easy to define whereas others had an indeterminate outline. Indeed, there appeared to be several morphological types of lobule. The categories for counting being divided into groups of 10 lobules each - and in the highest categories, 50 lobules - is acknowledgement of the fact that, in some instances, even after examination under high magnification, it is impossible to make precise separations between lobules.

Slices of tissue, therefore, were assessed independently by five observers to check the reproducibility of the counts. The slices were also assessed from both sides to determine whether the results remained unchanged and thus confirmed the transparency of the slices. The results from the five observers were comparable, indicating that, in the Edinburgh laboratory, there was general agreement as to what constituted a single lobule. The second difficulty was the long time required to count every cm^2 cell for all the slices in a breast. In an attempt to determine whether the time factor could be reduced for a second series of specimens, the effect of counting only alternate slices was assessed. This showed that counting half of the breast was not always representative of the

whole organ and indicated that there could be significant differences in lobule numbers from slice to slice of breast tissue. The graphs plotting lobule numbers against slice numbers for each quadrant revealed clearly that, in some breasts, the lobule numbers varied considerably from slice to slice as well as from one quadrant to another. Lobule numbers for all four quadrants combined initially increased from tissue adjacent to the nipple to that at the base of the breasts. However, in all of the breasts quantified, the slices immediately adjacent to the chest wall did not contain most lobules and the overall impression gained was that lobule numbers were at their peak at the junction of the middle and lower thirds of the breasts.

As only 10 breasts were quantified in the preliminary study, recording the individual variation between breasts, between quadrants in any one breast and from slice to slice of breast tissue, no firm conclusions could be drawn. Further investigation was indicated to clarify the following:

1. Whether the UOQ does or does not consistently contain the greatest number or density of lobules in the breast.
2. Where in the depth of the breast the greatest concentration of lobules lies.
3. Whether lobule number is related to breast size and/or the distribution of the NFC.
4. Whether there is any relationship between lobule number and other factors such as age, parity and laterality.
5. Whether, after examination of more surgical mastectomy specimens, the tumour quadrant is associated with the greatest number of lobules.

Counting only half of the breast proved inadequate to estimate correctly its lobule content and a modification of the quantitation procedure had to be devised to diminish the labour involved, thereby enabling a second series of specimens to be examined in the time available.

Quantitation of the Non-Fatty Component

In Table 6 it has been noted that there is a discrepancy between some of the readings recorded for the area of the NFC after fixation in formaldehyde and the areas recorded after staining and dehydration. This is of such a magnitude that in five of the breasts the differences between the values in the two sets of readings alters the order in which the quadrants are placed relative to one another in terms of the quantity of the NFC. As application of a Wilcoxon sum rank test showed that neither set of results was consistently larger or smaller than the other, it was speculated that the method of measuring the NFC was not sufficiently accurate and the protocol was reassessed to seek a possible explanation.

It is suggested that the difference between the two readings is due largely to the fact that, after staining the slices of breast tissue, there is in some specimens a better contrast between the NFC and the fat, even although the NFC does not take up the haematoxylin stain. This makes the margins of the NFC easier to record than in tracings taken after fixation, at which stage the lines of demarcation between the NFC and the fat can be difficult to distinguish.

In Table 7, the values obtained for the percentage of total tissue occupied by the NFC are larger after staining than after fixation in 30 out of the 40 quadrants examined. This result is significant using a Wilcoxon sum rank test ($p < 0.01$) and reflects the observation already made that there is a discrepancy in the two sets of readings recorded for the area occupied by the NFC.

However, a factor involved in all three tables is the shrinkage of the specimens during processing which was estimated to be approximately $5\frac{1}{2}\%$. Applying a Wilcoxon sum rank test to the figures for total area in Table 5, showed that, in 29 out of 40 quadrants, there was a decrease in the total area recorded after staining and this was significant ($p < 0.001$). It should be recalled that application of a Wilcoxon sum rank test to the data for the NFC in Table 6, showed that there was no significant difference between the readings after fixation and after staining. These findings indicate that fat shrinks to a greater extent than the NFC during processing. Shrinkage of the fatty component therefore also contributes to the wide variation seen in the figures in Table 7, and gives basis for a theory that the variation in visualisation of the margins of the NFC largely accounts for the difference between the two sets of readings.

The largest quadrant was the UOQ in 6 of the breasts examined, a figure which included all 5 of the left breasts. It may be argued that, in the other 4 specimens, removal of the breast at mastectomy or autopsy was incomplete in the axillary tail region and

this has been reflected in a low measurement for the UOQs of these breasts. It may also be argued that unrecorded lobules and NFC in the axillary tails has similarly influenced the results for these respective assessments. However, all axillary tail tissue removed for diagnostic purposes was stained and cleared. Neither lobules nor any measurable proportion of the NFC were found in any of the axillary tails examined.

Moreover, examination of tracings and slices of breast tissue revealed that, in the UOQ of 7 of the breasts, the margin of the NFC lay well within the fatty periphery of the slices. By superimposing the tracing for the NFC in a slice of tissue over the lobule quantitation results for the same slice, the two assessments could be compared as they were both to the same scale (Fig. 3.6). Comparison of a number of slices taken at random from each breast showed that the majority of lobules lay within the area occupied by the NFC.

It is unlikely that a bulk of NFC sufficient to affect the results in terms of both the NFC and lobules recorded in the UOQ was left behind on removal of the breasts at mastectomy or autopsy. In Table 5 it can be seen that the quadrant with the largest total area per breast coincided with the quadrant having the most lobules per breast in 5 out of the 10 specimens. Reference to the figures for the NFC in Table 6 shows that the quadrant with most lobules per breast coincided with the quadrant containing the most NFC in 6 of the 10 breasts. The quadrant containing the most NFC and total area per breast matched in 5 out of the 10 specimens examined.

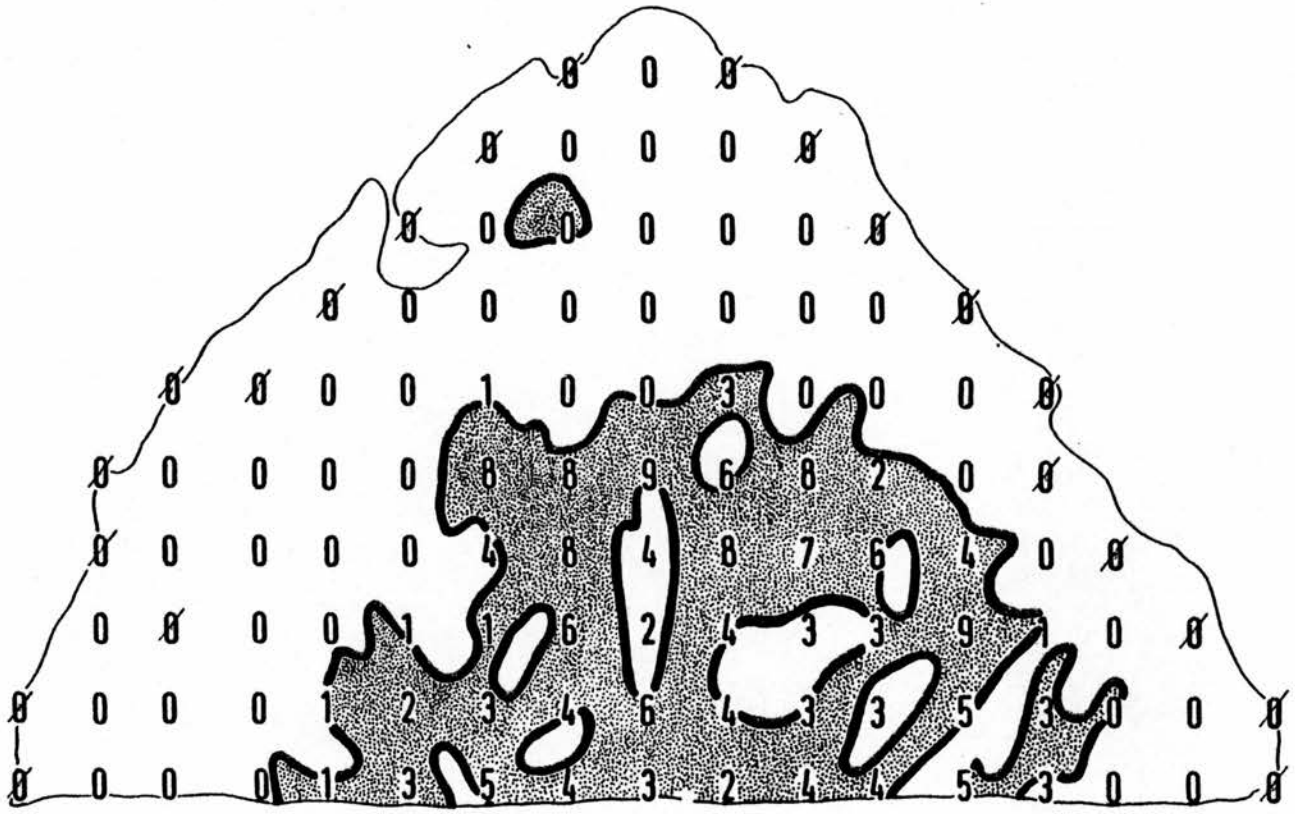


Fig. 3.6 Diagram to illustrate a tracing of the non-fatty component (NFC) in a coronal slice of breast tissue bisected in a sagittal plane superimposed over the results for quantitation of parenchymatous lobules within the same slice of tissue.

The largest breasts did not therefore necessarily contain the most lobules. Neither was there a significant relationship between the NFC and lobule number. Furthermore, the largest quadrant contained the most NFC in only 50% of the breasts examined.

From the results obtained it was hoped that it could be shown that the time and labour involved in future quantitative work could be reduced by counting only a proportion of the slices and thereafter estimating lobule number in the rest of the slices after measuring their total areas only. It was decided that the measurements obtained for both total area and the NFC would be examined in this manner to confirm whether the impressions gained from the graphs were correct.

STATISTICAL ANALYSIS

Attention was concentrated on the interpretation of the results from the preliminary study and, in particular, on the reduction of the time-consuming quantitative procedures for both the lobule and the NFC assessments. Graphs were plotted to examine the relationship between lobules and the NFC as well as between lobules and the total area for each quadrant of the 10 breasts. Measurements of the NFC both after fixation and after staining were separately plotted. The majority of graphs showed a positive relationship between lobule number and total area but not such an encouraging relationship between lobule number and the NFC. Examples are illustrated in Figs. 3.7 and 3.8.

From the results obtained, it was hoped that it could be shown that the time and labour involved in future quantitative work could be reduced by counting only a proportion of the slices, and forming an estimate of lobule number from values obtained for the total area. After counting the lobules in a minimum number of slices in a breast and plotting these results against the values obtained for the total areas obtained for all the slices, it was proposed to examine whether a straight line could be fitted through the points on the resultant graph. It was decided that both the NFC measurements and total area measurements would be examined in this manner to confirm whether the impressions gained from the graphs were correct. It was important, therefore, to calculate the correlation coefficients for graphs which plotted the total area and the area of the NFC against lobule number in order to ascertain how closely the

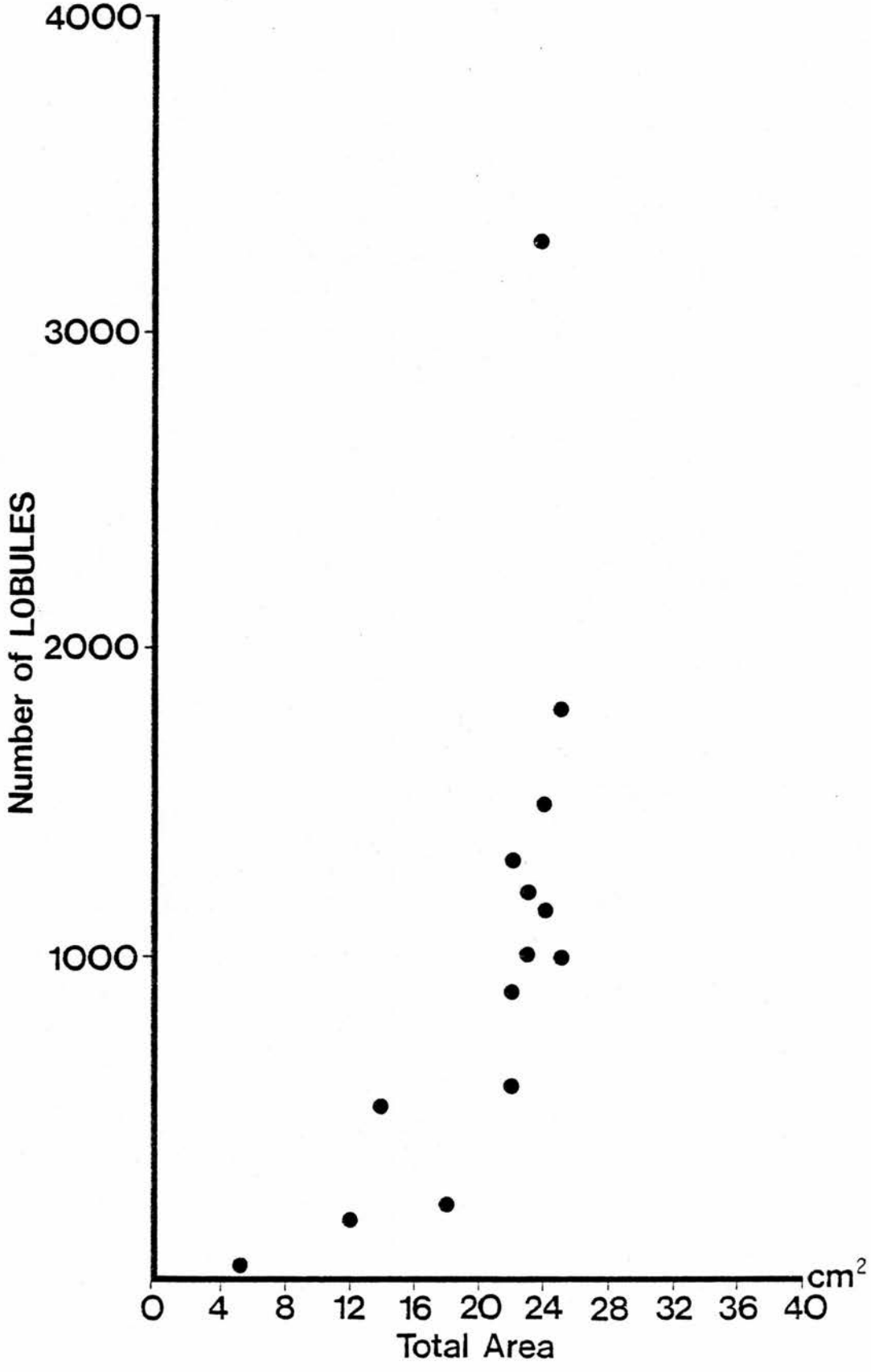


Fig. 3.7 Graph to illustrate the relationship between the total area and the number of parenchymatous lobules in the tissue slices of a whole breast (19 years).

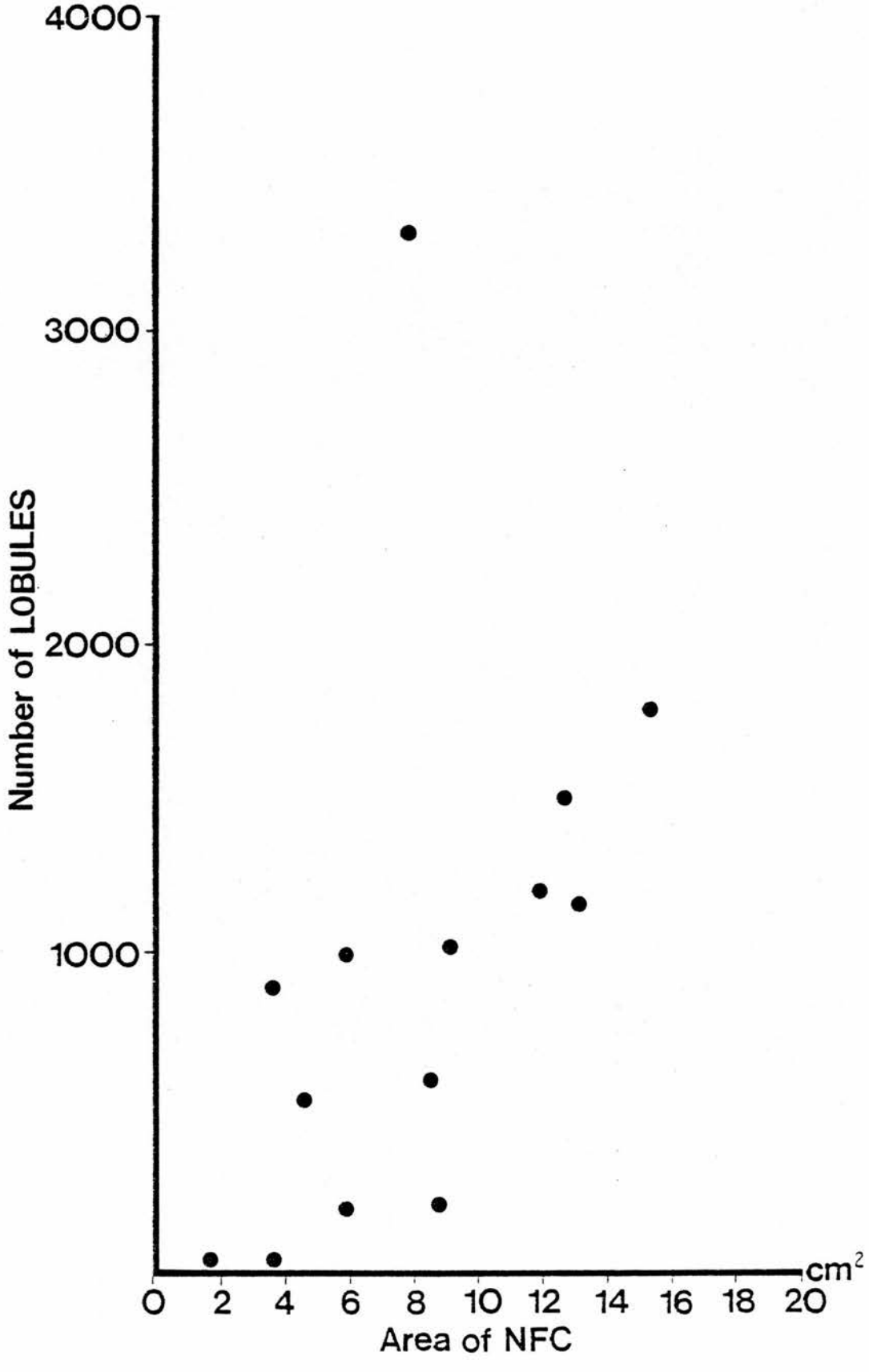


Fig. 3.8 Graph to illustrate the relationship between the area of the non-fatty component (NFC) and the number of parenchymatous lobules in the slices of tissue from the same breast as in Fig. 3.7.

components correlated. On an assumption that the lobules and the NFC are found occurring together, and that if there is no NFC there are no lobules, for correlation to be 100% the slopes of the graphs should pass in a straight line through the origin.

For calculation of the correlation coefficients, which were calculated for both total area and area of the NFC against lobule number, a small computer programme written in Fortran and run interactively on the ICL 2980 of the Edinburgh Regional Computing Centre, was devised. The correlations are those appropriate for a line forced through the origin, i.e. not the usual Pearson correlation coefficient. The programme allowed the input of pairs of x (area of NFC or total area) and y (number of lobules) values for each slice of tissue. For all four quadrants separately in each breast, the values for consecutive slices were entered into the computer which was programmed to measure the following from the x,y pairs:

a) number of data entered, i.e. number of slices

b) the total number of lobules

c) the estimated slope or density of lobules/cm^2 total area and of lobules/cm^2 NFC. This was calculated from

$$\frac{\text{total lobules}}{\text{total area of NFC}} \quad \text{and} \quad \frac{\text{total lobules}}{\text{total area slices}}$$

d) the estimated correlation measure for a slope forced through the origin (Deming, 1964).

The best estimate of the slope constrained to go through the origin is obtained by a weighted - least - square analysis which leads to the simple estimator

$$\hat{d}(\text{estimated density}) = \frac{\text{total lobules}}{\text{total area or area of NFC}}$$

The appropriate correlation coefficient for this model is

$$\frac{\Sigma\ (\text{lobules})}{\sqrt{(\Sigma\ \text{area})\left[\Sigma\ \frac{\text{lobules}^2}{\text{area}}\right]}}$$

This value is always positive as the presence of lobules is always positive. It has a maximum value of 1 which is only achieved when all the points lie on a straight line through the origin. It can be used to compare the strength of the linear relationship in different breasts.

e) A standard deviation of the density, weighted in favour of the largest values, given by

$$\sqrt{\frac{\Sigma\ (\text{density of } i^{th} \text{ slice} - \text{av. density})^2\ \text{Area } i^{th} \text{ slice}}{\text{total area or area of NFC}}}$$

From the results obtained it was possible to make an estimate of the standard errors of measurement for the calculated total number of lobules when only a proportion of the slices are counted. This was achieved by assuming a simple mathematical model of the variation in density of lobules from slice to slice. The best estimate of a slope constrained to go through the origin is then obtained (see d). The details of the mathematical model were provided by Mrs G.M. Raab (Medical Computing & Statistics Unit, University of Edinburgh) and may be consulted in Appendix B, V.

It was found that the correlations between lobules and total area were considerably stronger than between lobules and the area

of the NFC and the latter results are therefore not included. Tables 8-11 display the results for the correlation of number of lobules with the total area of the slices recorded after fixation, and after staining. Also in the tables are the figures for the coefficients of variation if only 50% of the slices are counted.

The formula for the coefficient of variation of the estimated total lobules is:

$$\left[\frac{\text{Weighted s.d. density}}{\text{density}}\right] \frac{1}{\sqrt{n-1}} \sqrt{\frac{\Sigma A_j}{\Sigma (A_J + A_i)} \left[1 + \frac{\Sigma A_j}{\Sigma A_i}\right]}$$

where (n-1) is the total number of slices in the breast, A_j is the sum of the areas of the slices not counted and A_i is the sum of the areas of the slices counted.

It should be noted that, if all the slices are counted, the coefficient of variation becomes zero and, if none are counted, it tends to infinity. It was necessary to discover whether the coefficients of variation when 50% of the slices are counted are sufficiently small to leave the conclusions unchanged. Should this be the case, the labour involved in quantifying the lobules would be considerably reduced.

From Tables 8-11 the number of lobules in each quadrant of the breast was converted into a percentage of the total number of lobules in the breast. The coefficients of variation (CV) were divided by 100 to express them as a proportion and two standard deviations (2 s.d.) calculated as below. Two standard deviations were used because 95% of the results will lie within ± 2 s.d. which is therefore a more sensitive measure than ± 1 s.d., within which 64% of the results will lie.

$$+ 2 \text{ s.d.} = \% \text{ lobules} \quad \left[1 + 2 \left(\frac{CV}{100}\right)\right]$$

$$- 2 \text{ s.d.} = \% \text{ lobules} \quad \left[1 - 2 \left(\frac{CV}{100}\right)\right]$$

The figures obtained were plotted in graphical form (Figs. 3.9 - 3.12). The purpose of these graphs was to illustrate whether it would have been effective to have counted only 50% of the slices in the breasts in this study. For distinction between the quadrants there must not be an overlap between the values for $\pm$ 2 s.d. It can be appreciated from the graphs that in no breast would it have been possible to ascertain the relationship between the quadrants in terms of lobule number by assessing only 50% of the slices.

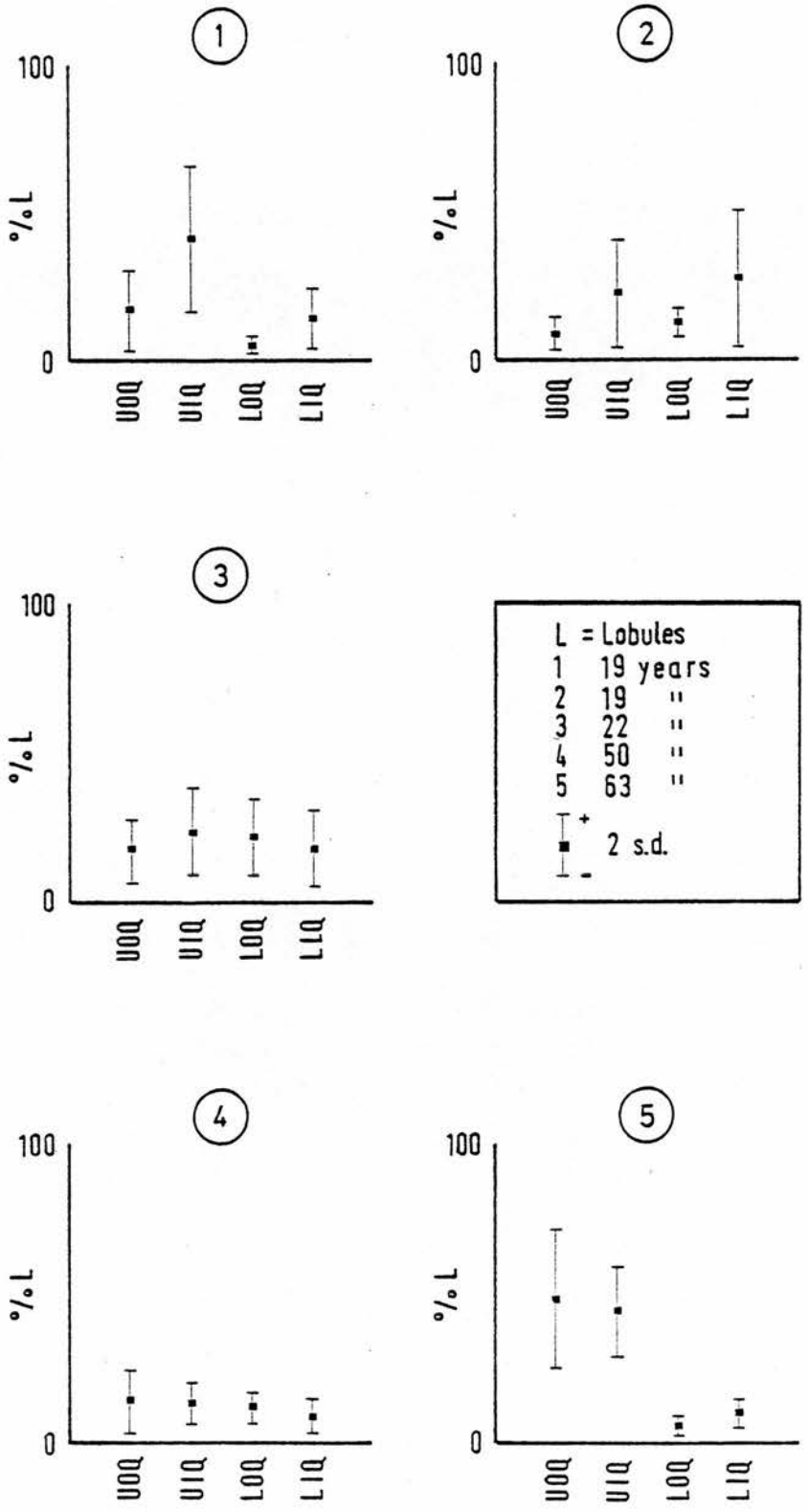


Fig. 3.9 Coefficients of variation due to estimating lobule count from 50% of total area in the quadrants of right breasts after formalin fixation.

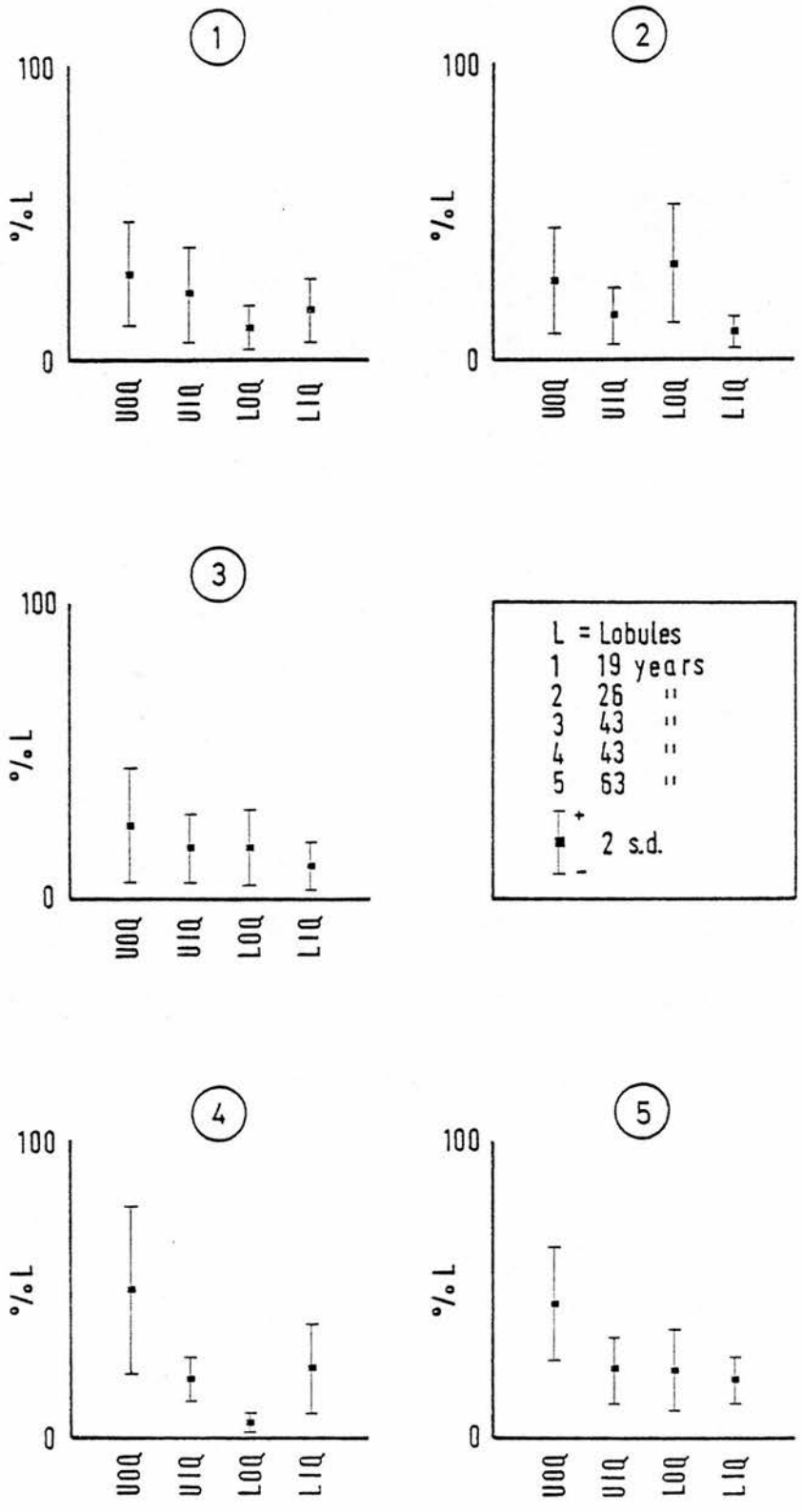


Fig. 3.10 Coefficients of variation due to estimating lobule count from 50% of total area in the quadrants of left breasts after formalin fixation.

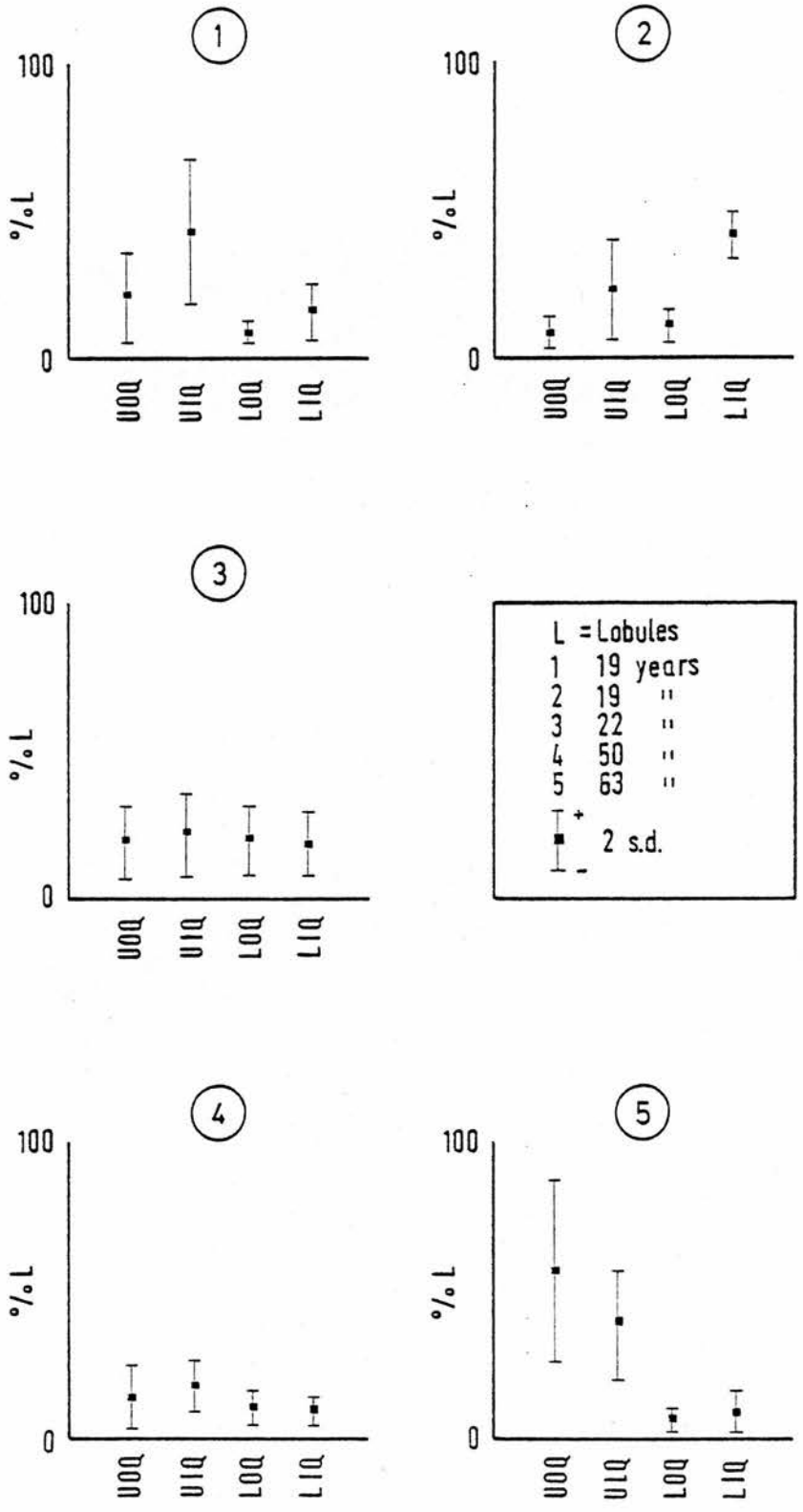


Fig. 3.11 Coefficients of variation due to estimating lobule count from 50% of total area in the quadrants of right breasts after staining with Delafield's haematoxylin.

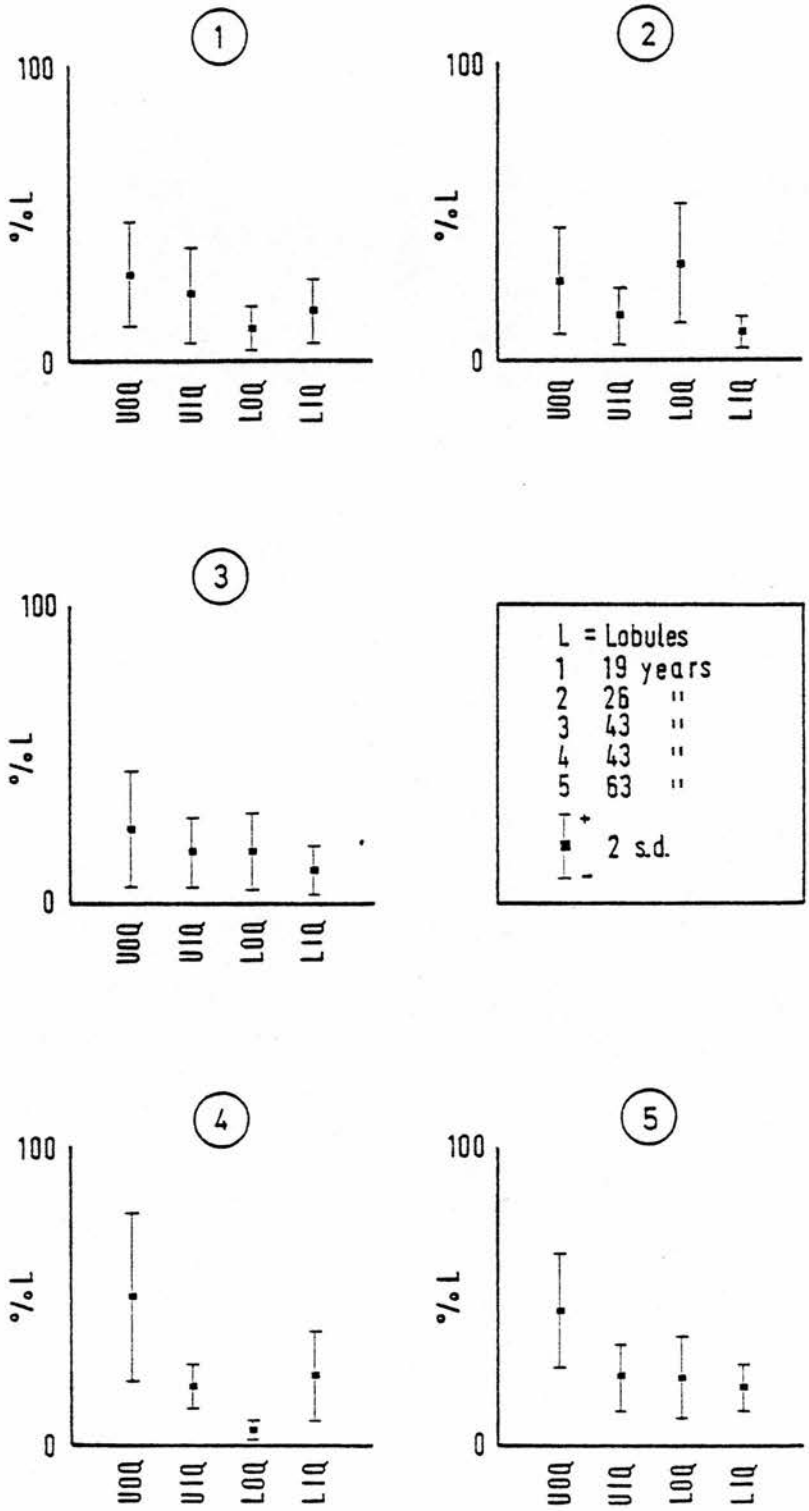


Fig. 3.12 Coefficients of variation due to estimating lobule count from 50% of total area in the quadrants of left breasts after staining with Delafield's haematoxylin.

MAIN STUDY

QUANTITATION OF LOBULES

As it had been ascertained in the preliminary study that considerably over 50% of the whole breast required to be assessed in order to determine accurately its contents and to distinguish one quadrant or area of the breast from another, it was decided that the simplest modification to the quantitation procedure was to reduce the number of categories counted, whilst still assessing the whole organ.

Materials

Whole breasts were obtained from autopsy suites and operating theatres as in the preliminary study. In the main study, 20 breasts were examined and details of these are listed below.

Age (in years)	Parity	Quadrant of tumour involvement	
Right breasts			
*21	-	-	
*25	-	-	
29	1+0	LOQ)	
29	1+0	LOQ)	
41	3+2	LIQ)	surgical
42	2+0	UOQ)	mastectomy
54	6+0	LOQ)	
*56	3+0	UOQ)	

Age (in years)	Parity	Quadrant of tumour involvement	
Left breasts			
*21	-	-	
22	-	-	
*25	-	-	
25	0+0	UOQ + LOQ)	
30	2+0	UIQ)	
31	2+0	UOQ)	
35	0+0	Nipple)	surgical
38	2+0	LIQ)	mastectomy
39	0+0	LOQ)	
41	0+0	UOQ)	
55	2+1	UOQ)	
*56	3+0	-)	

* Matching pairs of right and left breasts.

Methods

The breasts were stained and cleared in the same manner as for the preliminary study. To facilitate microscopic examination, instead of the slices of breast tissue being submerged in methyl salicylate in an open glass pot, they were individually sealed in polythene bags (Kapak Corporation, Bloomington, USA). These bags, being completely transparent, were suitable for use with a microscope and also minimised fumes arising from the methyl salicylate.

To quantify the lobules, an acetate grid of 1 cm^2 cells was attached with sellotape to each bag containing a slice of tissue. Orientation of the grid to divide the breast tissue into quadrants was as previously described in the preliminary study. Every cm^2 cell containing breast tissue was assessed. As before, lobules falling on the lines of the acetate grid were included in the count for the cell within which most of the lobule was contained. Cells having less than half of their area covered by breast tissue were

noted. All the slices in each of the 20 breasts were assessed. The 14 categories of the preliminary study were reduced to the following:-

Category	Number of lobules
0	0
1	1- 34
2	35- 70
3	71-100
4	over 100

These categories effectively made an assessment of very high, high, medium and low densities of lobules. The highest category, i.e. 4, combined categories 11 to 14 in the preliminary study encompassing a range of 100 to 250 lobules. As the initial survey showed that there are relatively few areas in a breast that contain over 100 lobules per cm^2, this large upper range was considered acceptable. The reduction in categories greatly decreased the time required for the quantitation procedure and made the counting of a further 20 breasts feasible.

As by using categories containing large numbers of lobules, there could be an element of bias involved in taking the midpoint figure as the number of lobules representing that category, a lobule score was therefore used to present the results. This acts as a measure of lobule density rather than actual lobule number, and also accommodates the criticism that it can be difficult to separate lobules from one another. The lobule score for each slice was derived by weighting the results for each category as follows:-

Lobule category	Weighting for each category
1	x 1
2	x 2
3	x 3
4	x 4
	Total = lobule score per slice

Cells near the periphery of slices, which most commonly were those recorded as being less than half full of tissue, were observed to contain in nearly all cases very low lobule numbers. As each lobule category represented much larger numbers of lobules than was the case in the preliminary study, the lobule scores in cells less than 50% full of tissue were halved to avoid bias.

The total number of cells assessed for each slice was also calculated and represented an estimate of total area. Again, the number of cells recorded as being less than 50% full of tissue was halved to avoid bias. The average lobule scores per cm^2 were then able to be calculated.

Results

For each of the 20 breasts quantified, the results of the slices were combined to give the lobule scores (i.e. estimates of lobule number), the total number of cells counted and the average lobule scores per cell for the 1) whole organ; 2) each quadrant; 3) outer and inner halves; 4) upper and lower halves; 5) combinations of the UOQ + LIQ and the UIQ + LOQ; 6) superficial, middle and deep thirds. The results are presented in Appendix C, Tables 12-20. Statistics

were used to examine the distribution of lobule scores in the breasts as well as the effects of age, parity, tumour involvement and laterality on the results.

All statistical tests applied to the results were performed using every specimen and then, to avoid bias, repeated eliminating one of each pair of breasts.

Distribution of lobule scores in the quadrants of the breast

The lobule scores for each quadrant (Table 12) were compared using the Friedman test which showed that the quadrants in the series of specimens were statistically different from each other ($p < 0.01$). This finding indicated that the data should be examined in greater detail to determine whether any one quadrant had consistently higher lobule scores than the other quadrants.

Consequently, Wilcoxon sum rank tests were used to compare the lobule scores between the four quadrants of the breasts. The same tests were also used to compare the lobule scores in the upper with the lower halves of the breasts; the outer with the inner halves and the LOQ + LIQs with the UIQ + LOQs. The statistical results are presented in Table 21.

The statistical results indicated that the UOQ did not significantly contain the highest lobule scores. Table 21 shows that there was no statistical difference between the lobule scores recorded in the UOQ and the UIQ, or between those recorded in the LOQ and the LIQ. Both the UOQ and the UIQ contained higher lobule scores than the LOQ. The upper half of the breast contained higher

lobule scores than the lower half. The outer half of the breast was not statistically different from the inner half. The UOQ + LIQs combined contained significantly higher lobule scores than the UIQ + LOQs combined. The results indicated that there was an eccentric distribution of lobules with a bias towards the upper half of the breast.

The average lobule score per cm^2 cell of tissue examined was calculated for each quadrant in each of the 20 specimens (Table 14). Applying the Friedman statistic to these results showed that there was no significant difference between the four quadrants of the breasts. The average lobule scores were also calculated for the outer and inner halves; upper and lower halves; as well as for the UOQ + LIQs and the UIQ + LOQs combined (Table 17). Application of the Friedman test again showed that there was no significant difference between any of the sets of figures.

Effect of age

The Spearman's statistical test was applied to compare the total lobule scores for the 20 whole breasts with age in years. It was found that lobule numbers significantly decreased with increasing age in years ($p < 0.02$). The Spearman's test applied to compare the average lobule score per cm^2 cell of tissue with age in years showed that the density of lobules did not significantly decrease with age in years.

Effect of parity

The effects of parity on the results were examined. The Spearman's test was used to compare total lobule scores as well as

average lobule scores for the whole breasts in parous and nulliparous cases. No significant differences were found. Within the parous group, lobule scores were compared with increasing numbers of pregnancies both including and excluding miscarriages. The number of pregnancies was not significantly related to lobule score.

Effect of biopsy site

Mann-Whitney U tests were applied to the results for lobule scores to compare the scores for quadrants which contained a biopsy site and/or residual tumour with the scores obtained for "normal" quadrants. The tests were repeated using the average lobule scores per cm^2 cell of tissue. No relationship emerged indicating that quadrants with a history of carcinoma displayed significantly higher or lower lobule scores than normal quadrants.

Effect of laterality

Mann-Whitney U tests were used to compare the results for lobule score as well as for average lobule score per cm^2 cell of tissue for both the whole organ and the UOQ in right and left breasts. No statistical significance was revealed for any of these comparisons.

Comparison of paired specimens

As only 3 pairs of breasts were included in the lobule quantitation, application of statistics would not have been meaningful. Observation of the results indicated that neither right nor left side consistently contained most lobules.

Distribution of lobules in the superficial, middle and deep thirds of the breast

Nineteen breasts were included in this assessment as one specimen had been sliced sagitally during processing and therefore could not be divided into superficial, middle and deep thirds.

Wilcoxon sum rank tests were applied to the results in Table 18 to compare the distribution of lobule scores in the superficial, middle and deep thirds of the breasts. The tests revealed (Table 22) that the highest lobule scores occurred in the middle third of the breast. The lobule scores for the superficial and deep thirds were not significantly different. Lobule scores were therefore not found to be consistently highest at the base of the breast.

The average lobule scores occurring per cm^2 cell of tissue were calculated for the superficial, middle and deep thirds of the breast (Table 20). Wilcoxon sum rank tests applied to these results confirmed that lobules were found in highest concentrations in the middle third of the specimens. Once again there was no significant difference between the results for the superficial and deep thirds of the breasts.

QUANTITATION OF THE NON-FATTY COMPONENT

Materials

Breast specimens were obtained from autopsy suites and operating theatres as in the preliminary study. In the main study 30 breasts were examined and details of these are listed below.

Age (in years)	Parity	Quadrant of tumour involvement	
Right breasts			
19	-	-	
*21	-	-	
*22	-	-	
*25	-	-	
29	1+0	LOQ)	
30	2+0	UIQ)	
37	4+2	UIQ)	
38	4+0	UOQ)	mastectomy specimens
40	3+0	LIQ)	
41	3+2	LIQ)	
42	2+0	UOQ)	
*56	3+0	UOQ)	
*63	3+0	-)	
Left breasts			
19	-	-	
*21	-	-	
*22	-	-	
25	0+0	UOQ + LOQ)	
*25	-	-	
35	0+0	Nipple)	
41	0+0	UOQ)	
44	3+0	UIQ)	
46	0+0	UOQ)	
46	1+1	UOQ)	
46	2+1	UOQ)	mastectomy specimens
53	0+0	UOQ)	
55	2+1	UOQ)	
*56	3+0	UOQ)	
57	1+0	UOQ + LOQ)	
*63	3+0	-)	
73	0+0	UOQ)	

* Matching pairs of right and left breasts.

Methods

After fixation and slicing of each breast was completed, radiographs were taken of all the slices of tissue in the breast. Using transmitted light provided by a transparency viewer (Kodak Ltd., Glasgow, Scotland), tracings were made of the periphery of each slice as well as the boundaries of the NFC within each slice. From the tracings the total area of each slice and the area occupied by the NFC in each slice was measured using an image analyser (GDSI 4051; Tektronix UK Ltd., Herts., England).

Results

The results were expressed as the area occupied by the NFC in cm^2, the total area in cm^2, and the percentage of NFC per slice of tissue. Areas were not converted to volume measurements as this would have added the variable of slice thickness, and it therefore was considered that the breasts were more accurately compared using area rather than volume.

For each of the 30 breasts examined, the results for the slices were combined to give figures for the 1) whole organ; each quadrant; 3) outer and inner halves; 4) upper and lower halves; 5) combinations of the UOQ + LIQ and the UIQ + LOQ; 6) superficial, middle and deep thirds. The results are presented in Appendix C, Tables 23-30.

Statistical treatments (the Friedman statistic, Mann-Whitney U test, Wilcoxon sum rank test and Spearman rank correlation tests) were applied to the results for both the total area and the area of

NFC to examine their distribution as well as the effects of age, parity, laterality and tumour involvement on the results. The tests were applied to the results for all 30 breasts measured and then to the results after the elimination of one of each pair of breasts, i.e. a total of 25 specimens. Thus, each breast was individually treated and any bias incurred by including both breasts from one patient was avoided.

Comparison of quadrants

Using the Friedman statistic on the results for the area of NFC and the total area in Tables 24 and 25, the four quadrants of the breast were found to be significantly different from each other ($p < 0.001$).

To determine the order in which the quadrants and selected areas of the breasts may be placed in terms of total size and content of the NFC, Wilcoxon sum rank tests were applied to the results in Tables 24, 25 & 27-29. The results of the statistics are summarised in Table 31 and they suggest that, in spite of individual variation, the NFC can be described in general terms as an ellipse in shape with the UOQ containing the greatest area of NFC. The results for total area and the results for the area of NFC match well and significance levels were not altered by eliminating one of each pair of breasts.

This matching of results for the total area and the area of NFC raises the interpretation that the NFC content of a breast is proportional to total area and that, therefore, the anatomical finding that more NFC is found in the UOQ exists simply because this

quadrant is generally the largest. To test this hypothesis the percentage of total area occupied by the NFC was calculated for each quadrant (Table 26). The Friedman test was applied to the percentages and it was found that, when all 30 or - eliminating one of each pair - 25 breasts were tested, the quadrants were not significantly different from each other. This finding supports a hypothesis that the NFC of the breast is related to total area of tissue.

The average percentage of NFC per quadrant with standard errors (s.e.) is quoted below:-

For 30 breasts:

UOQ	UIQ	LOQ	LIQ
43.0±4.32 s.e.	36.31±3.30 s.e.	43.67±4.08 s.e.	38.15±3.60 s.e.

For 25 breasts:

UOQ	UIQ	LOQ	LIQ
42.09±4.41 s.e.	34.33±3.23 s.e.	43.10±4.20 s.e.	36.83±3.61 s.e.

Biopsy sites

It is important to consider whether the inclusion of quadrants containing any tumour and/or biopsy sites has biased the results. The presence of a tumour and/or biopsy site in a specimen could have the following effects:

a) The size of the biopsy taken may be large and therefore the NFC subsequently measured may have been significantly depleted by the "missing" tissue.

b) Granulation tissue at a biopsy site may show as a radio-opacity and be included on tracings as NFC.

c) The presence of a tumour may increase artificially the area of the NFC measured and may also have an effect on the total NFC in a quadrant that is unknown.

Details of the sizes and sites of all biopsies, which had been taken prior to mastectomy, were obtained from case histories (Appendix C, Table 3). Examination of the data recording the NFC showed that it is very unlikely that the significance of the results for the order of quadrant content has been affected by either biopsy size or the presence of granulation tissue.

Residual tumour

In only 6 breasts was there any residual tumour evident in the mastectomy specimen. In these breasts the tumour quadrant was separated from the quadrant with the nearest result to the tumour quadrant by many more cm^2 of NFC than the tumour could have occupied as a radio-opacity. Out of the 20 surgical mastectomies with histories of carcinoma, in only 9 cases does the quadrant having tumour origin match the quadrant with the most NFC. Mann-Whitney U tests used to compare the percentages of NFC in normal quadrants and quadrants with a history of tumour were not significant.

Effect of age

Spearman's rank correlation tests applied to the raw data for the NFC indicated that the quantity of NFC decreased with increasing age in years ($p < 0.02$). However, the tests applied to the results

for the percentage incidence of the NFC compared with age in years were not significant.

Effect of parity

Mann-Whitney U tests were used to compare the results for both the NFC and the percentages of NFC obtained from parous and nulliparous specimens. Within the parous group, using Spearman's rank correlation tests, the NFC and the percentage incidence of the NFC were compared with increasing numbers of pregnancies, both including and excluding miscarriages. None of these tests proved to be of statistical importance.

Effect of laterality

Results for the NFC and the percentage of NFC obtained for the whole organ as well as for the UOQ were compared for right and left specimens using Mann-Whitney U tests. There was no significant difference between the results either for the whole organ or for the UOQ.

Comparison of paired specimens

Wilcoxon sum rank correlation tests applied to the results for the 5 paired specimens included in this study indicated that there was no significant difference in the NFC or the percentage incidence of NFC between pairs.

Contraceptive pill usage, menstrual cycle status and family history of carcinoma

Of the women from whom the 40 breasts which were subjected to quantitation procedures were derived, only 6 were using a contraceptive

pill preparation and, of those for whom menstrual cycle status was known, the majority were in the luteal phase. Four women gave a positive family history of carcinoma. Ten women had undergone a hysterectomy or were menopausal and 10 breasts were autopsy specimens. There were not enough data within each category to enable a statistical evaluation of any one of these factors using the results for either the NFC or lobules to be made.

Distribution of non-fatty component in the superficial, middle and deep thirds of the breast.

The results in Table 30 were examined by Wilcoxon sum rank tests to compare the areas of NFC found in the superficial, middle and deep thirds of the breast. A total of 29 breasts was included in this analysis, as one breast was sliced in a sagittal plane and therefore could not be divided into superficial, middle and deep thirds. The statistical results are presented below. All 29 breasts were included in the statistics, and the test repeated eliminating one of each pair, to make a total of 24 specimens.

	NFC 29 breasts	p	NFC 24 breasts	p
Deep third > middle third in	12	NS*	12	NS*
Deep third > superficial third in	19	< 0.01	17	< 0.01

* Not significant

In only 19 out of 29 breasts, or 17 out of 24 breasts, was the content of the NFC in the deep third of the breast greater than in the superficial third. Breasts also varied as to whether they showed

greater areas of NFC in the deep or middle thirds; and in only 12 out of 29 and 12 out of 24 specimens was most NFC recorded in the deep third.

DISCUSSION

This study has been performed in an attempt to assess both the parenchymal and connective tissue contents of whole breasts. There does not appear to be another study in the literature in which such a project has been undertaken. The majority of other workers who have attempted to quantify the human breast have done so using histological sections obtained from biopsy material (Richardson, 1952; Munford, 1964; Underwood, 1972; Wilson, 1976; Nienhaus & Brenner, 1977; Theele & Bässler, 1981; Drife, 1982).

The overriding impression emerging from this study is that the breast is, not unexpectedly, a very individual organ for which it is difficult to generalise and lay down rules. To understand completely the distribution of the components in any one specimen, the entire organ must be studied. This of course is a time consuming operation which inevitably results in a sample of moderate size. Further, as the breast has the added complication of superimposed variables such as age, parity and menstrual cycle status which may influence results, it is difficult on examination of limited numbers to draw conclusions that may be in any way definitive.

It is not only in the human situation that the individual nature and variation of the breast is a feature. Cowie (1947), in his

quantitation of the rat mammary gland, stated that all the glands in any one animal should be assessed as the "marked" difference in development noted by previous workers between the glands from rats of the same age and even between corresponding glands of either side in the same rat has been confirmed.

A statistical trend emerged in this study indicating that the NFC was related more closely to total breast area than to lobule number. Although the NFC could be idealised as having an elliptical form, with the greatest area of tissue occurring in the UOQ, the results also revealed that the UOQ was not always the quadrant containing most lobules. Neither the most lobules nor the most NFC was related in all the specimens to the largest quadrant size, nor indeed to each other; and neither the NFC nor the lobular distribution was even between the quadrants. Also, the largest breasts did not necessarily contain the greatest quantities of either NFC or lobules and this finding endorsed the work of Engel (1941) as well as the classifications of breast type (e.g. Sandison, 1962) which are commonly used. From the results it would not be a valid assumption that the NFC of the breast is pyramidal or cone-like in shape, the base of the cone (and therefore the most NFC) coinciding with the base of the breast. The distribution of the NFC in the breast was different from that of the lobules which had an eccentric distribution with a bias towards the upper half of the breast.

Lobules frequently occurred in areas of high focal concentration and were consequently capable of altering markedly in number from slice to slice of tissue. They tended to occur in greatest numbers at the junction of the middle and lower thirds of the breast. The NFC

also appeared to concentrate in the middle third, contributing to an impression that the stromal and parenchymal elements lie within the fatty tissue of the breast in a rounded or ball-shaped manner rather than in a conical fashion.

The finding that lobule number decreased with increasing age is as expected when the physiology and changes of the menopause are taken into account. It is, however, an interesting observation that the density of lobules/cm^2 tissue, as well as the total number of lobules, can be the same in old and young women. Parity did not influence the results for either the lobule or the NFC assessments.

There appeared to be no correlation between lobule or NFC quantity and tumour incidence within quadrants. The results suggested that tumours do not arise more commonly in the UOQ because there is more tissue in this quadrant, and that it cannot be predicted whether the largest quadrant will contain either the most NFC or lobules. However, as over a large series of specimens it is most likely that this will be the case, the incidence of tumours arising at random within the parenchyma may therefore show a higher correlation with the UOQ than with other regions of the breast.

Referring to Garfinkel *et al.* (1959), who questioned whether there was more breast tissue in left than right breasts, the results in this study do not support such a theory. However, the sample of 5 pairs of breasts is small and is therefore difficult to evaluate statistically as a separate group.

It can be argued that lobule size has not been taken into sufficient account in this project as both lobule number and size

contribute to total parenchymal quantity. Lobule number reflects the number of terminal ducts and consequently the ducts themselves, which are also a component of the parenchyma, were not separately quantified. From observation and random measurements it was noted that lobule size in any one breast was surprisingly uniform within that breast and variation was not sufficient, even although differing subgross lobule "types" may be present in areas of tissue, to affect a comparison of quadrants. Individual breasts appeared to display a dominant lobule appearance in terms of lobule type.

It is significant that whole breasts have been quantified as this provides a base line from which alternative approaches may be assessed; and the fact that breasts show such variation within themselves makes alternative approaches complex. Other methods of quantitation which have been considered in this research include histological and ultra-structural stereological analysis (Chalkley, 1943; Weibel & Elias, 1967; Dunnill, 1968; Aherne, 1970; Mayhew & Williams, 1971); computer card assessments (Winsberg *et al.*, 1967; Meinhard, 1974; Meinhard, Wadbrook & Risdon, 1975); quantimet analysis (Nienhaus & Brenner, 1977); graphic stereo-reconstruction of serial sections (Yamada & Yoshida, 1972; Payne & Hutchins, 1973); the use of densitometers and microspectrometers and a variety of methods which have been applied to other organs and animals (Short, 1950, 1952; Campbell & Tomkieff, 1952; Dunnill, 1968; Boatman & Lowe, 1971; Risdon, 1974). All these techniques rely on the use of histological sections and do not offer the advantages of a three dimensional total assessment. It has been shown that counting the lobules in half of the breast is not sufficient to differentiate between quadrants or to

predict with a degree of certainty the total lobule content. It should therefore be questioned whether assessments making use of biopsies are valid for conclusions relating to the whole organ.

Having established the capability for variation in the distribution of both the NFC and the parenchyma in the breast, it is suggested that, with the exception of a Teutonic piece of research, the utilisation of biopsy material is unlikely to reveal whether one breast contains more parenchyma or fibrous connective tissue stroma than another, let alone provide the specificity required to distinguish one quadrant from another.

SUMMARY

The content of lobules and the NFC in whole breasts has been quantified. Neither lobules nor the NFC were evenly distributed between the four quadrants of the breast and the UOQ did not contain significantly greater numbers of lobules than the other quadrants. Lobules were found occurring in foci of high concentration and were most numerous at the junction of the middle and deep thirds of the breast. No significant differences were detected in the quantity of either lobules or the NFC between right and left sides, both for the whole organ and for the UOQ. There was no significant difference between the results obtained for tumour and non-tumour quadrants; or for parous and nulliparous breasts. The number of lobules and the size of measurements obtained for the NFC decreased significantly with age, but their distributions were not related to each other. It was determined that, in order to quantify the lobules in the whole breast so that quadrants might be differentiated from one another, over 50% of the whole organ must be assessed owing to variation occurring between slices of tissue. Thus, the questions posed at the end of the preliminary study have been answered.

CHAPTER 4

MORPHOLOGY OF SUBGROSS LOBULE TYPES IN THE BREAST

INTRODUCTION

In studies involving subgross stereomicroscopic assessment of breast tissue, considerable emphasis has been placed on the recognition of pathological and potentially pathological lobule types (Wellings & Jensen, 1973; Wellings, *et al.*, 1975; Jensen & Wellings, 1976; Jensen, Rice & Wellings, 1976; Brem, Jensen & Gullino 1978; Sarnelli *et al.*, 1980a,b). There have been few studies on the existence of a range of lobule types within the normal specimen at a three dimensional level (Wellings, 1980; Vogel *et al.*, 1981). 'Within normal limits' is a term commonly used in studies of the breast. Histologically, it is a description often applied to lobules which cannot be placed in any category of pathology and which frequently accompanies a diagnosis of benign mammary dysplasia. In the present study, the lobule types most often found in what is generally regarded as normal breast tissue are described as 'normal' lobules and four types have been recognised. They have distinctive subgross appearances and may be observed with ease using a stereomicroscope. A series of stained and cleared breasts encompassing a wide age range has been investigated in an attempt to establish how these subgross lobule types vary in their incidence within specimens of the same age and with increasing age. Other factors considered which may act to alter the lobule type and appearance include successive menstrual cycles with their attendant hormonal influences, laterality, parity and increasing numbers of pregnancies, and the use of a contraceptive pill preparation. Many atypical, precancerous

and cancerous subgross parenchymal appearances have been described (e.g. Wellings, *et al.*, 1975; Wellings *et al.*, 1976; Sarnelli *et al.*, 1980a,b), but in the present study only the incidences of cysts, apocrine cysts and fibroadenomata were assessed. The aim of the investigation was to determine whether these lesions occur over a wide age range, in both nulliparous and parous breasts and also in both normal breasts and "normal" tissue derived from mastectomies.

In order to extend the examination of normal lobule types in the breast, duct injection techniques followed by slicing, staining and clearing procedures have been performed and the lobules associated with a single lactiferous duct system examined for their range of variation. Further, a comparison has been made between the radiographic and the subgross appearances of slices of tissue in an attempt to establish whether lobule type and abundance may be predicted from radiographic appearances alone.

There is extensive literature on the subject of mammograms and their interpretation. The pioneer work on mammography was performed by Salomon (1913), Warren (1930), and Fray & Warren (1932). Since their studies many researchers have concentrated on the interpretation of pathological lesions seen on radiographs, but seldom have workers stated how they classify the normal appearances of the breast on a mammogram. Authors offering basic classifications of normal include Lockwood & Stewart (1932), Lockwood (1934), Hicken (1937), Leborgne (1953), Ingleby & Gershon-Cohen (1960), Egan (1970, 1972), Wolfe (1976a,b), and Wolfe & Wilkie (1978).

Leborgne (1953) describes the normal radiological anatomy of the breast as falling into three areas: the corpus mammae (made up of fibrous tissue, glandular elements and ducts), the adipose tissue and the skin. He states that it is the fibrous tissue which in 'physiological repose' constitutes the greater part of the corpus mammae and, for correct visualisation of the glandular elements, they must be made opaque by the use of contrast media.

Ingleby & Gershon-Cohen (1960) describe the breast in a different manner. Trabeculae which run in the breast from the base of the organ to the nipple are conspicuous on radiographs and are composed of both stroma and parenchyma. In "glandular breasts during the reproductive period", lobules occupy the base and periphery of the breast and the trabeculae. Only lactiferous ducts and clusters of fingerlike offshoots are found in the subareolar area.

Wolfe's classification (1976a,b) describes radio-opacities in terms of parenchyma. He regards the breast as being composed of a) fat; b) connective tissue and epithelial elements (which may be seen as dysplasia on the mammogram) and c) periductal collagenosis (which is seen as a prominent duct pattern). His hypothesis, that two of four mammographic patterns show a marked predisposition to the development of breast cancer, has been supplemented by further work (e.g. Wolfe, 1979; Wolfe *et al.*, 1982). A number of other studies have also associated the mammographic patterns he describes with varying degrees of risk of the development of breast cancer (Wilkinson *et al.*, 1977; Brebner, Epstein & Lange, 1978; Hainline *et al.*, 1978; Krook, 1978; Krook *et al.*, 1978; Chaffe, Roebuck & Worthington, 1979; Threatt *et al.*, 1980; Boyd *et al.*, 1982;

Brisson *et al.*, 1982a). Other workers, however, deny that the radiographic appearance of the breast can determine the risk of developing carcinoma (Egan & Mosteller, 1977; Mendell, Rosenbloom & Naimark, 1977; Rideout & Poon, 1977; Fisher *et al.*, 1978; Moskowitz, Gartside & McLaughlin, 1980; Egan & McSweeney, 1979). Several studies have reported that increases in age (Wolfe, 1976c; Peyster, Kalisher & Cole, 1977; Moskowitz *et al.*, 1980; Threatt *et al.*, 1980) or in parity (Ernster *et al.*, 1980; Gravelle *et al.*, 1980) are associated with a large reduction in the percentage of the breast showing nodular and homogeneous densities on mammograms which are indicative of the high risk parenchymal patterns described by Wolfe (1976b). Brisson *et al.* (1982b) observed that the age at delivery of the first full term child and the family history of breast cancer showed little relation to the mammographic features assessed. However, inconsistent results have been obtained for both these factors (Wilkinson *et al.*, 1977; Andersson, Andrén & Pettersson, 1978; Krook, 1978; Ernster *et al.*, 1980).

Recently, Tabár & Dean (1982) have stated that, if the findings of Wolfe are valid for the general population and if they really can be applied to individual cases, then their value would be enormous in the diagnosis and management of breast diseases. But they stress the need for confirmation of Wolfe's results based on the study of a large non selected population undergoing mammography, a view shared by other workers including Check (1980). Tabár & Dean (1982) state that this is particularly necessary since some of the conclusions of Wolfe are being "applied to the general population and have already influenced clinical practice". In their own research they were unable

to corroborate Wolfe's work and conclude that the parenchymal patterns "should not be used to influence patient management or screening programmes". Moskowitz *et al.* (1980) also state that, in their experience, "too many people are now planning patient management based on these patterns". Egan (1964) reminds readers that "just as has every diagnostic procedure, mammography has its limitations, and errors are made." No significant correlation between types of histopathological alterations and mammographic densities as described by Wolfe was found in specimen mammograms by Fisher, Posada & Ramos (1974); Fisher *et al.* (1978) and the subject remains controversial because of the many equivocal results reported.

It is apparent from the literature that the interpretations of exactly what is being seen on mammograms are different, e.g. what Leborgne describes as fibrous tissue strands, Ingleby & Gershon-Cohen describe as trabeculae and Wolfe describes as ducts. Lockwood & Stewart (1932) describe menstrual cycle changes on normal radiographs making clear distinction between parenchyma and stroma. Egan (1972), however, in describing the normal breast at varying times of life, states that three major types of breast tissue are seen on mammograms. These are fibrous, glandular and adipose tissues; and, since the fibrous and glandular tissues have nearly the same effective atomic number or $\overline{Z}$ number, i.e. 7, they cannot be separated adequately on mammograms. Therefore he concludes that the term 'fibroglandular tissue' best describes these structures on the mammograms. Adipose tissue with a $\overline{Z}$ number of 6 is more radiolucent and provides contrast. It is the relative amounts of fat and fibroglandular tissue that characterise primarily the breast types

and influence the diagnostic accuracy of mammography. Having stated this, his subsequent descriptions frequently involve direct reference to glandular tissue appearances and, in his diagram illustrating the various anatomic structures of the breast which can be identified on the mammogram, the bulk of the radio-opacity is described as glandular tissue, within which are trabeculae.

The authors quoted above represent a small selection from an enormous literature on the subject of mammography. Radiographic techniques have been extended from diagnostic examination of the breast prior to surgery to examination of the pathological specimen following mastectomy. Also, radiography is now being employed to examine the comparatively small volumes of tissue obtained from breast biopsies (Eastgate, Gilchrist & Matallana, 1979; Philip, Harris & Rustage, 1982). Studies using radiographs of serial slices of breasts coinciding with subgross and/or histological examination of the subsequently stained, cleared and processed tissue have been performed by numerous workers such as Ingleby & Gershon-Cohen (1960), Gallager & Martin (1969), Hutter & Kim (1971), Fisher, Dow & Posada, 1973; Fisher *et al.* (1978) and Wellings & Wolfe (1978); and it has been implied that parenchymal appearances may be predicted from or 'match' radiographic appearances. In the present study a comparison has been made between the radiographic and the subgross appearances of coronal slices of breast tissue in an attempt to establish whether lobule type and abundance may be predicted from radiographic appearances alone.

MATERIALS

Whole breasts were obtained from autopsy suites and operating theatres. The majority of female subjects at autopsy are post-menopausal and most premenopausal breasts had to be obtained from surgical excisions (mastectomies). A total of 47 breasts were examined, the details of which are listed below.

Age (in years)		Parity
Right breasts		
*19)		-
19)		-
*21)	autopsy	-
*25)		-
*50)		-
29)		1 + 0
29)		1 + 0
30)		2 + 0
34)		2 + 0
37)		4 + 2
38)		4 + 0
40)		3 + 0
41)	surgical mastectomy	0 + 0
42)		2 + 0
*44)		3 + 0
46)		2 + 0
*49)		3 + 0
*56)		3 + 0
63		3 + 0
Left breasts		
*19)		-
*21)		-
22)	autopsy	-
*25)		-
*50)		-
25)		0 + 0
26)		0 + 0
31)	surgical mastectomy	2 + 0
35)		0 + 0
38)		2 + 0

(contd.)

Age (in years)		Parity
Left breasts (contd.)		
39)		0 + 0
41)		3 + 2
43)		3 + 2
43)		3 + 0
*44)		3 + 0
46)		1 + 1
46)		2 + 1
46)		0 + 0
49)	surgical mastectomy	3 + 0
50)		4 + 0
50)		2 + 0
50)		1 + 0
53)		0 + 0
54)		6 + 0
55)		0 + 0
*56)		3 + 0
57)		1 + 0
73)		0 + 0

* Matching pairs of right and left breasts

METHODS

The breasts were sliced, stained and cleared according to the method described in chapter 2.

EXAMINATION OF TISSUE

Normal Lobule Types

Normal lobules observed as three dimensional structures in the cleared slices of breast tissue were divided into four categories

1. Solid lobules (Fig. 4.1)

Lobules having a smooth well circumscribed outline. The ductules composing the lobules are compactly arranged and have distinct lumina.

2. Feather-like lobules (Fig. 4.2)

Lobules which have very poorly defined boundaries. The ductules have no distinguishable lumina.

3. Microcystic lobules (Figs. 4.3-4.5)

Lobules with more than one-third of their ductules significantly dilated in comparison to the remainder of the ductules in those lobules. Microcysts usually have a diameter of less than 1 mm but may reach 3 - 4 mm (Parks, 1959).

4. Intermediate lobules

This category encompasses those normal lobules not falling into the above categories. Intermediate lobules form a spectrum of varying lobule appearances, and in this study have been divided into two types which are usually found occurring together:

Type A (Figs. 4.6 and 4.7): lobules composed of up to 10 ductules. The lobular outline is irregular and it can be difficult to distinguish one lobule from another. The ductules have distinct lumina and may be compactly arranged or spaced apart. Occasionally the ductules have a glove-like appearance and resemble blunt duct adenosis as described by Foote & Stewart (1945b).

Fig. 4.1

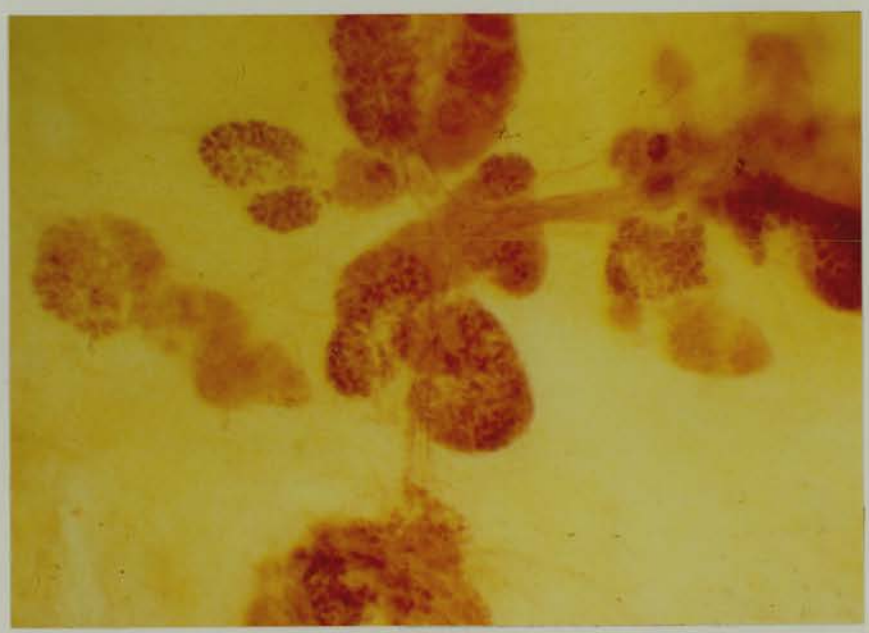
Field of cleared breast tissue (19 years). The lobules throughout the field are solid in type.

Delafield's haematoxylin. (Mag. x 18).

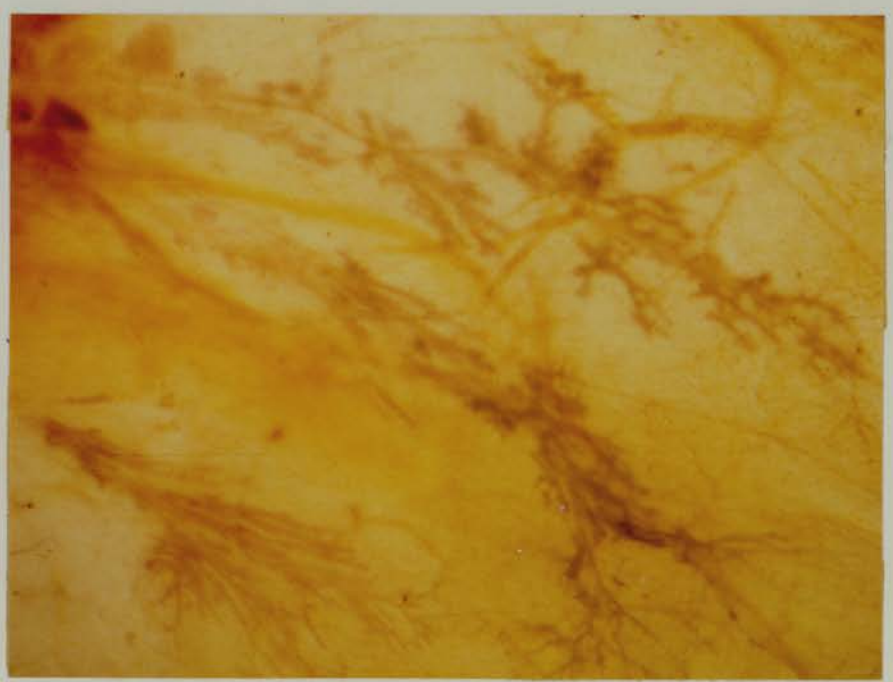
Fig. 4.2

Field of cleared breast tissue (44 years). The field is occupied by feather-like lobules having a structure which is indeterminate.

Delafield's haematoxylin. (Mag. x 12).



4.1



4.2

Fig. 4.3

Field of cleared breast tissue (38 years). In the top right of the field a few lobules are microcystic and have dilated terminal ductules.

Delafield's haematoxylin. (Mag. x 10).

Fig. 4.4

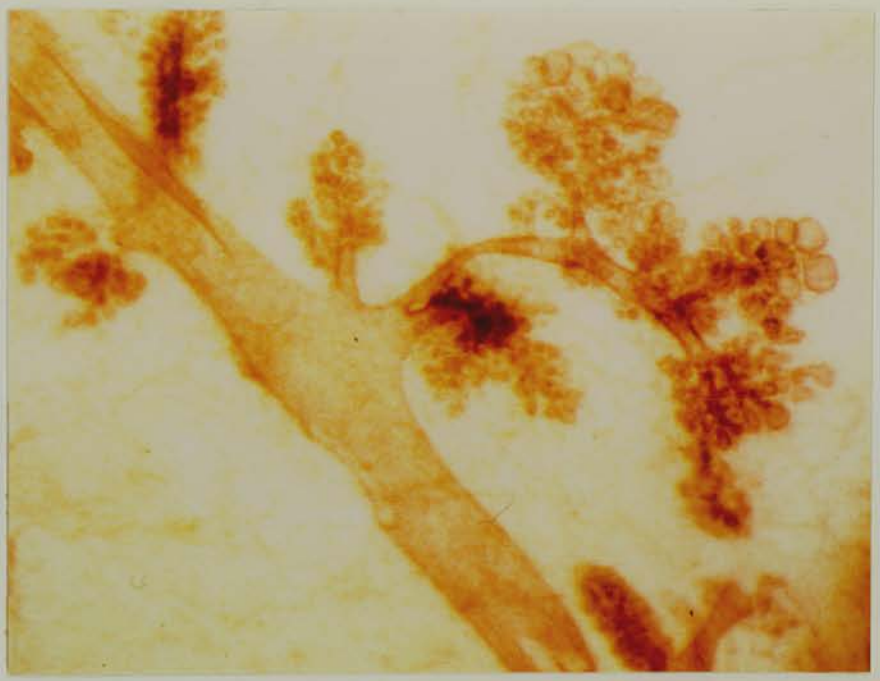
Field of cleared breast tissue (42 years). The majority of lobules are microcystic and have dilated ductules.

Delafield's haematoxylin. (Mag. x 18).

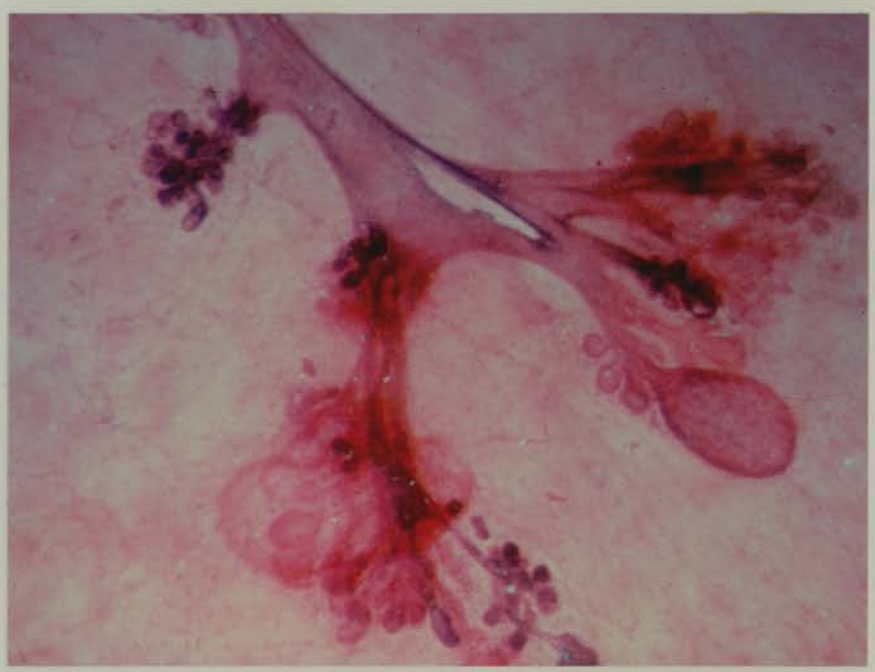
Fig. 4.5

Field of cleared breast tissue (56 years). The lobules are microcystic and have dilated ductules.

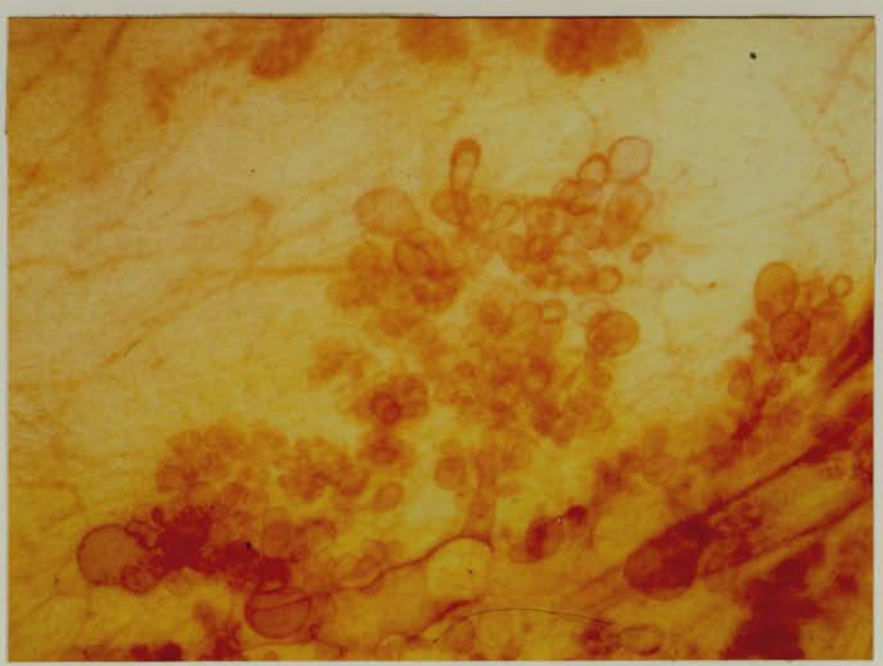
Delafield's haematoxylin. (Mag. x 12).



4.3



4.4



4.5

Fig. 4.6

Field of cleared breast tissue (35 years). In the left of the field are intermediate type A lobules. It is not easy to isolate these lobules for photography as they are not frequently found occurring in isolation from intermediate type B lobules. Delafield's haematoxylin. (Mag. x 10).

Fig. 4.7

Field of cleared breast tissue (22 years). The left of the picture shows blind ending ducts/ductules which were included in the category of intermediate type A lobules.
Delafield's haematoxylin. (Mag. x 30).

Fig. 4.8

Field of cleared breast tissue (50 years). Intermediate type B lobules are illustrated.
Delafield's haematoxylin. (Mag. x 20).

4·8

4·7

4·6

Type B (Fig. 4.8): lobules composed of more than 10 ductules. The lobular outline is irregular and the ductules may be spaced or compactly arranged.

Each slice of tissue in all of the 47 breasts was examined in the same manner. Using the Wild M8 stereomicroscope at x 6 and x 12 magnifications, the assessment of the breasts was performed in such a way that, out of a total of 100% per slice of tissue, a percentage was assigned to each lobule type to reflect its incidence in every tissue slice. No subdivisions of 5% intervals were used. The percentage obtained was not intended as an actual measurement but rather represented an estimate of the prominence which each lobule type showed in a slice of tissue. The lobule types were assessed systematically: solid, feather-like, microcystic and, finally, intermediate type lobules. To avoid bias, the breasts were coded so that relevant data such as age and parity were not known at the time of assessment. Slices selected at random were assessed by a second observer to ensure reproducibility.

Altered Lobules

As stated in the introduction, also noted in the study were parenchymal appearances which have been considered to be derived from altered or even pathological lobule types and these were placed in a category which included:

1. Individual and groups of cysts bigger than 4 mm and with no visible lobular association.

Fig. 4.9

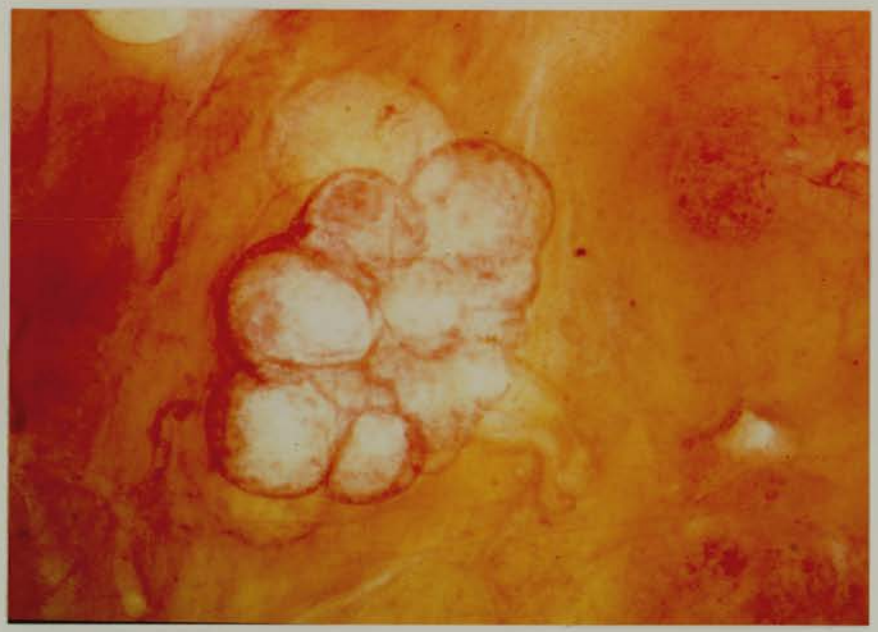
Field of cleared breast tissue (46 years). The centre of the field features a group of apocrine cysts.

Delafield's haematoxylin. (Mag. x 10).

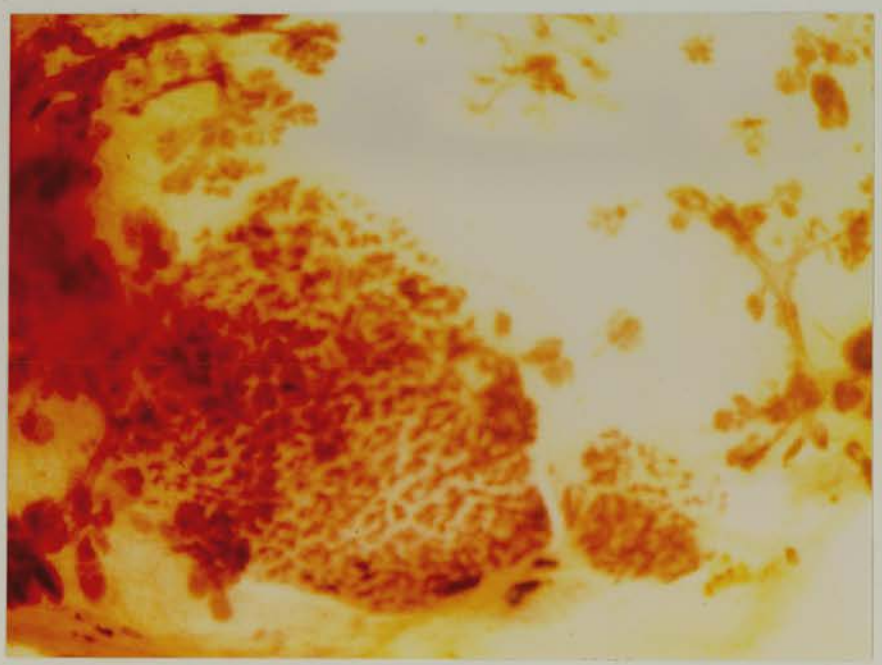
Fig. 4.10

Field of cleared breast tissue (29 years). To the bottom and left of the picture a small fibroadenoma is illustrated.

Delafield's haematoxylin. (Mag. x 6).



4.9



4.10

2. Individual and groups of cysts showing apocrine change (Fig. 4.9).

3. Fibroadenomata (Fig. 4.10).

The incidence of these individual and groups of altered lobule types was assessed as an actual count rather than as a percentage estimate per slice of breast tissue.

Duct Injection Techniques

Single duct injections were performed on four breasts according to the method described in Appendix A, VIII. Until intercommunications between lactiferous duct systems have been confirmed, it is assumed that by injecting one lactiferous duct per breast, all radiopaque material revealed in the subsequently sliced, stained and cleared specimens related to the terminal ducts and lobules of a single duct system. In this manner, differing lobule types (if any) relating to one duct system could be observed. For each breast, all the slices were examined with the stereomicroscope at low power (x 6; x 12) as well as at higher powers (x 25; x 40). Every slice of tissue was photographed and the photographs enlarged to scale so that the individual duct injections could be followed through the tissue from slice to slice. Details of black and white as well as colour photography are provided in Appendix A, XIV.

Radiographic Examination

Each breast in this study was radiographed as a whole specimen prior to processing as were all the tissue slices prior to staining and clearing. This radiographic examination had a twofold

purpose: firstly, it was used by the pathologist as a diagnostic aid and record, particularly for mastectomy specimens. Secondly, visual comparisons between the photographic and radiographic records were made for each case to ascertain whether subgross lobule type could be predicted from radiographic appearances alone. The details of exposure and use of radiographic film are listed in Appendix A, VII.

RESULTS

LOBULE MORPHOLOGY

Normal Lobules

The results for all the slices in a breast were totalled and a percentage obtained representing the incidence of each lobule type in the whole specimen. Table 32, Appendix C, shows the percentage incidence of each lobule type in the 47 whole breasts examined. All statistical treatments applied to the results are described in Appendix B. The tests were performed using every specimen and then, to avoid bias, repeated eliminating one of each pair of breasts.

Effect of Age

Using Spearman's rank correlation tests, the incidence of lobule types was compared with age and the following results obtained:

Solid lobules decreased with age	$p < 0.01$
Intermediate lobules increased with age	$p < 0.05$
Microcystic lobules increased with age	$p < 0.001$
Feather-like lobules increased with age	$p < 0.05$
Lobule types combined other than solid lobules increased with age	$p < 0.001$

The tests were re-applied to the results for those breasts included in age ranges of 19-40 years and 41-73 years to ascertain

whether the findings in either younger or older women were responsible for contributing more heavily to the overall significance. This was not found to be the case and neither group was in itself significant.

Effect of Parity

Mann-Whitney U tests were used to compare the results for parous with nulliparous breasts. The comparison was performed using the results in percentages for solid lobules as well as for the other lobule types combined. No significant difference was found between parous and nulliparous specimens using either set of results. Neither was any significant difference detected within the parous group taking into account increasing numbers of pregnancies. As most women are likely to have completed child-bearing by the age of 40 years, it was decided to re-apply the statistics to the results from women in the 19-40 years age range to eliminate any bias which older age groups may have imposed on the results. There was no significance obtained comparing parous with nulliparous results or between the results for increasing numbers of pregnancies within the parous group in women up to 40 years old.

Effect of Laterality

Mann-Whitney U tests were applied to the results from right and left breasts for solid lobules and other lobule types combined. None of the tests showed that laterality was of any significance in the incidence of lobule types.

Comparison of Paired Specimens

Results for the seven pairs of breasts included in this study were compared using Wilcoxon sum rank correlation tests. There was no significant difference in the results for either solid lobules or other lobule types combined between paired specimens.

Contraceptive Pill Usage, Menstrual Cycle Status and Family History of Carcinoma

It was considered unsatisfactory to analyse statistically the results with respect to these factors as it was not possible to separate them from the effects of age and/or there were insufficient data for comparison of results.

Altered Lobules

Spearman's rank correlation tests were used to examine whether the overall incidence of cysts and cysts showing apocrine change increased significantly with age. The tests were performed using the results (Appendix C, Table 33) for all 47 breasts and then, to avoid bias, after the elimination of one of each pair of breasts. The results for the incidence of both types of cyst were then combined and the tests repeated. The statistical findings are reproduced below:-

	47 breasts	40 breasts
cysts	$p < 0.001$	NS
apocrine cysts	$p < 0.05$	NS
cysts and apocrine cysts	$p < 0.001$	$p < 0.05$

*NS - not significant

To examine whether the number of breasts derived from young women in the sample were producing bias, Spearman's rank correlation tests were applied to the results from breasts of women aged 40 and above but did not show significance. Application of Mann-Whitney U tests ascertained that parity was not related to the results. The use of a Wilcoxon sum rank test determined that there was no significant difference between the results obtained for the pairs of breasts. Fibroadenomata were observed in only nine breasts and their infrequency rendered statistical analysis unmeaningful.

Radiographic Examination of Breast Slices

Figures 4.11a and 4.12a illustrate radiographs of breast slices of the same 2 mm thickness from two women of 21 and 22 years of age. Figures 4.11b and 4.12b illustrate the respective subgross appearances of the stained and cleared slices. Whereas both showed similar radiographic appearances, a marked difference can be observed in the parenchymal content of the two slices which were taken from approximately the same depth in the breast. The radiographs and stained and cleared slices of all 47 breasts were compared to see whether parenchymal content, subgross lobule type, age, parity, contraceptive pill usage or menstrual cycle status were related to the radiographic appearances of the breast slices. No consistent correlations were obtained and the overall impression gained was that the distribution of the non-fatty component appeared to be the most significant feature determining the pattern of radiopacity rather than the parenchymal quantity or quality. Examples of radiographs and cleared slices are shown in Figs. 4.13-4.16.

Individual duct injections were followed through stained and cleared slices aided by reference to corresponding radiographs. It proved very difficult to observe lobules containing micropaque connected to terminal ducts. However, terminal ductal-lobular units were isolated in a few instances and, although the identification of any detailed structure under the stereomicroscope was poor and not facilitated by the opacity of the micropaque, it appeared to be the case that more than one lobule type was associated with a single lactiferous duct system (Figs. 4.17 and 4.18).

Fig. 4.11

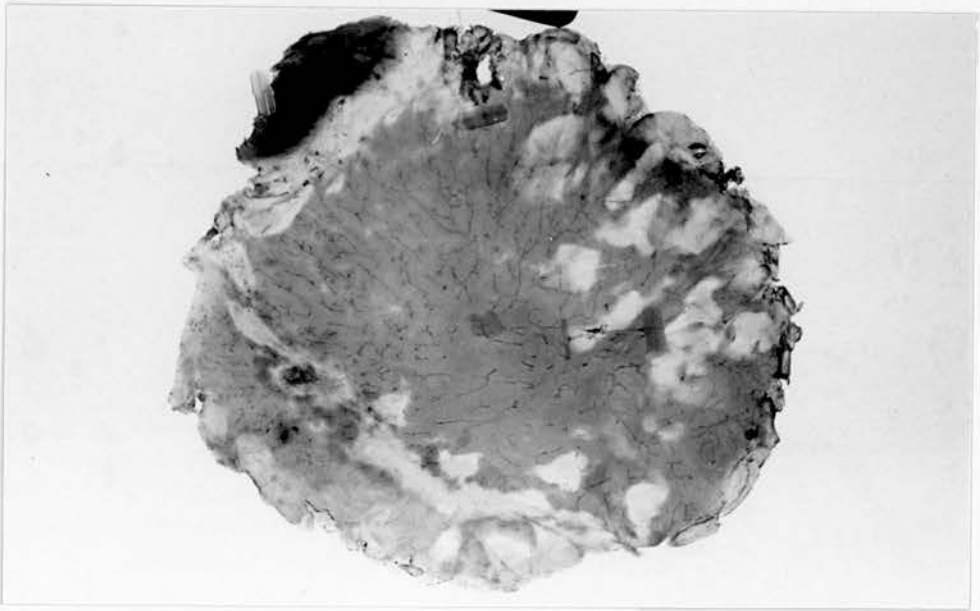
Coronal slice of breast tissue (21 years). Mag. x 0.5.

a) Radiograph

b) Stained (Delafield's haematoxylin) and cleared tissue.

Relative to the radiograph, the slice of tissue is rotated in this picture a little to the left. Very few parenchymatous elements may be observed and these are predominantly ducts.

4·11a



4·11b

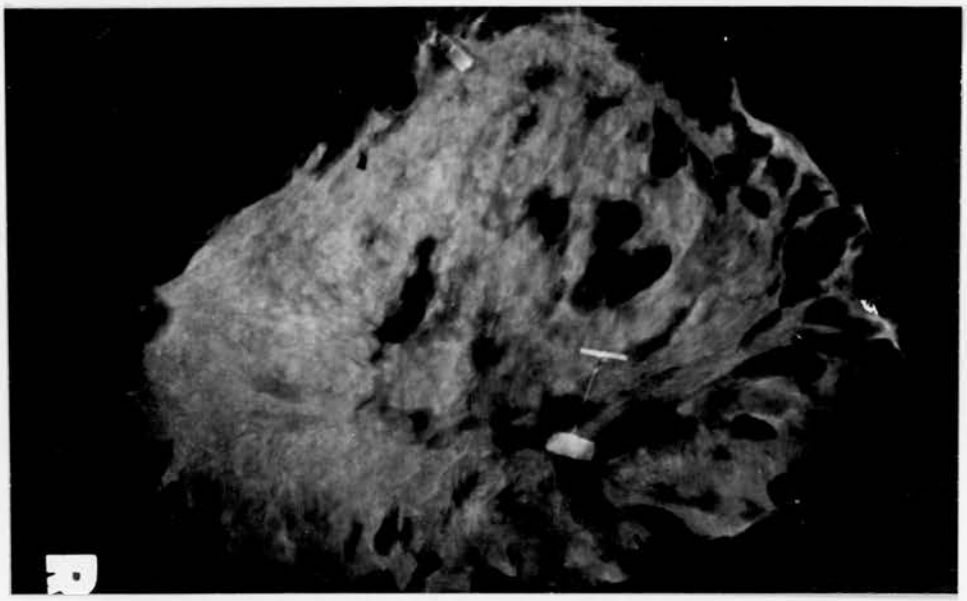
Fig. 4.12

Coronal slice of breast tissue (22 years). Mag. x 0.5

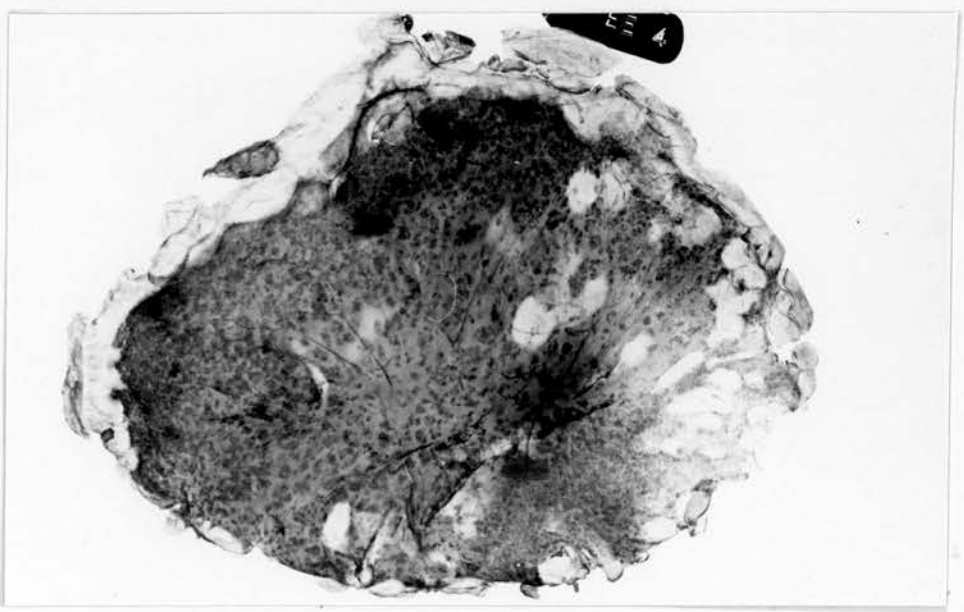
a) Radiograph

b) Stained (Delafield's haematoxylin) and cleared tissue.

A high concentration of parenchymatous elements, predominantly lobules are present.



4·12a



4·12b

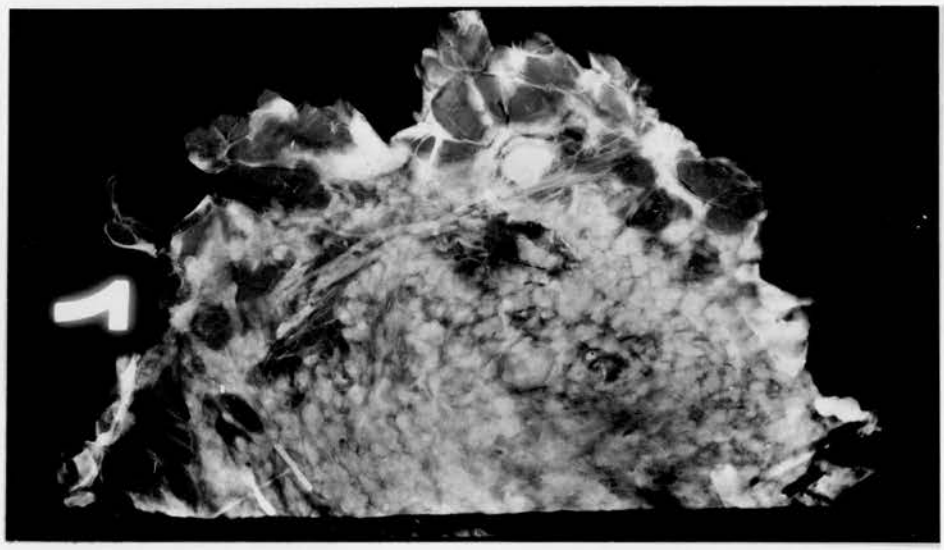
Fig. 4.13

Coronal slice of breast tissue bisected in a vertical plane (37 years). Mag. x 0.5.

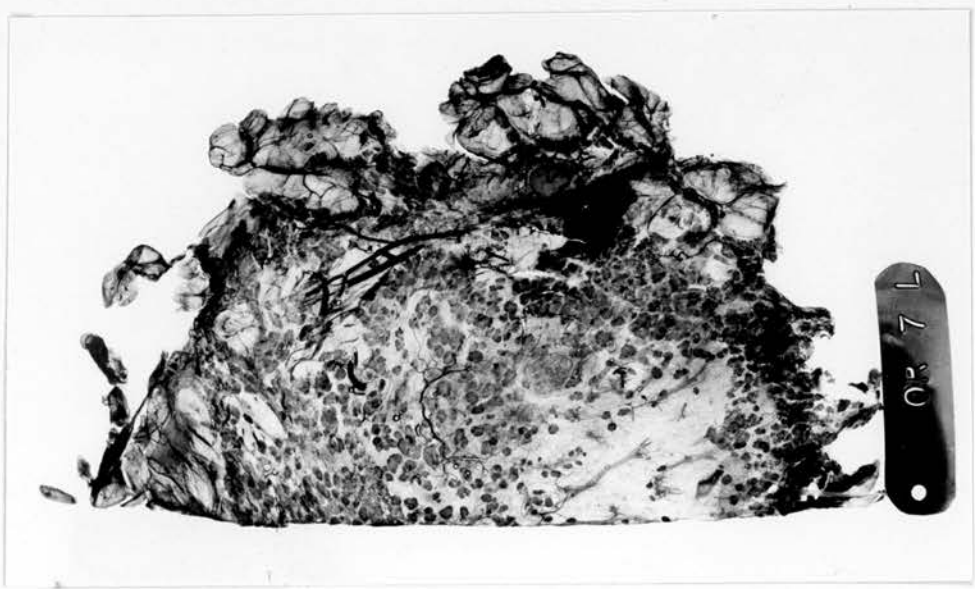
a) Radiograph

b) Stained (Delafield's haematoxylin) and cleared tissue.

Large and plentiful parenchymatous lobules may be distinguished and their positioning corresponds to the mottled radiographic appearance.



4·13a



4·13b

Fig. 4.14

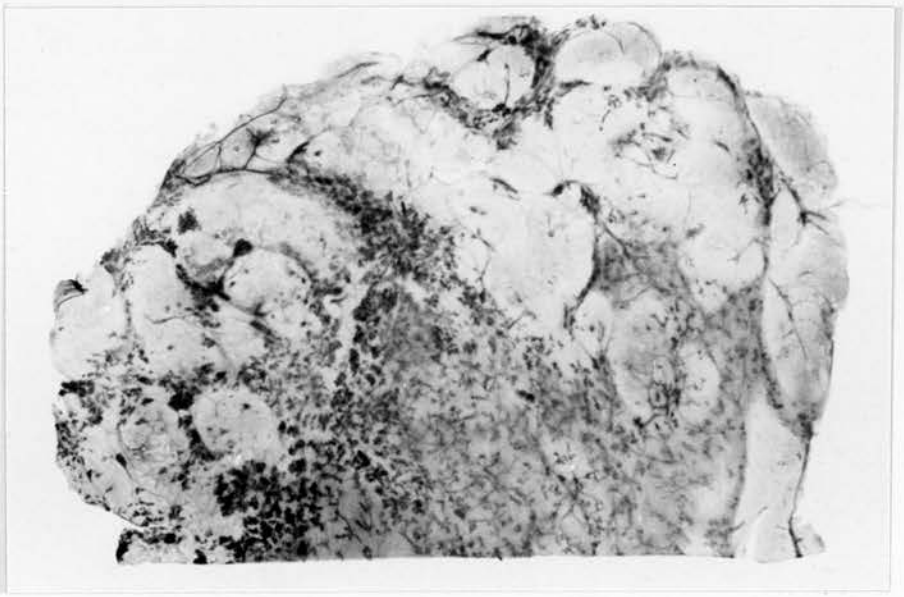
Coronal slice of breast tissue bisected in a coronal plane (19 years). Mag. x 0.6.

a) Radiograph

b) Stained (Delafield's haematoxylin) and cleared tissue.

The parenchymatous lobules are numerous and particularly evident to the left of the slice where the connective tissue is less dense and has been more completely cleared. Reference to the corresponding radiograph indicates that in this area the mottled appearance of the lobules is more easily distinguished.

4.14a



4.14b

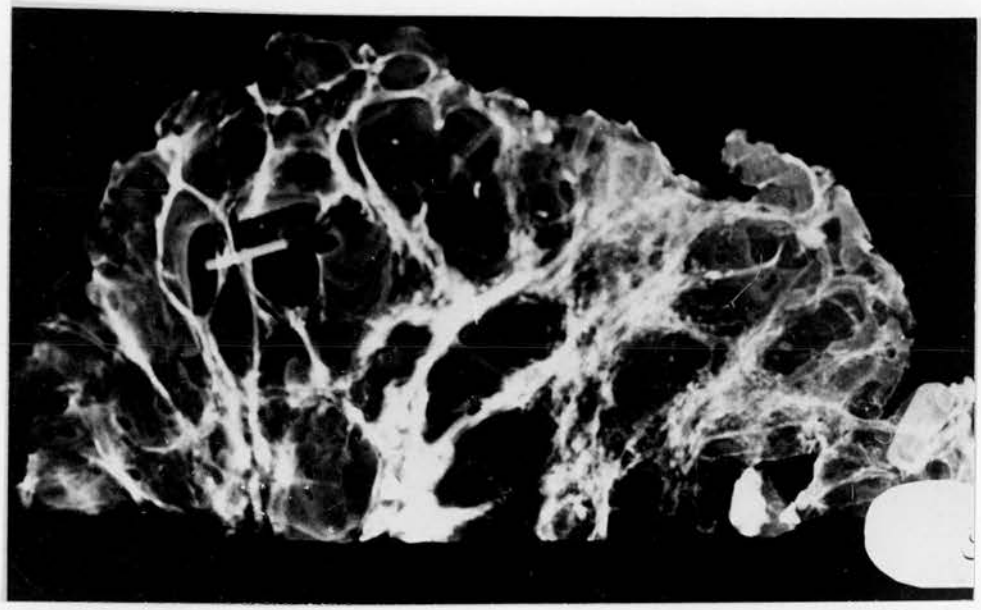
Fig. 4.15

Coronal slice of breast tissue bisected in a sagittal plane (46 years). Mag. x 0.5.

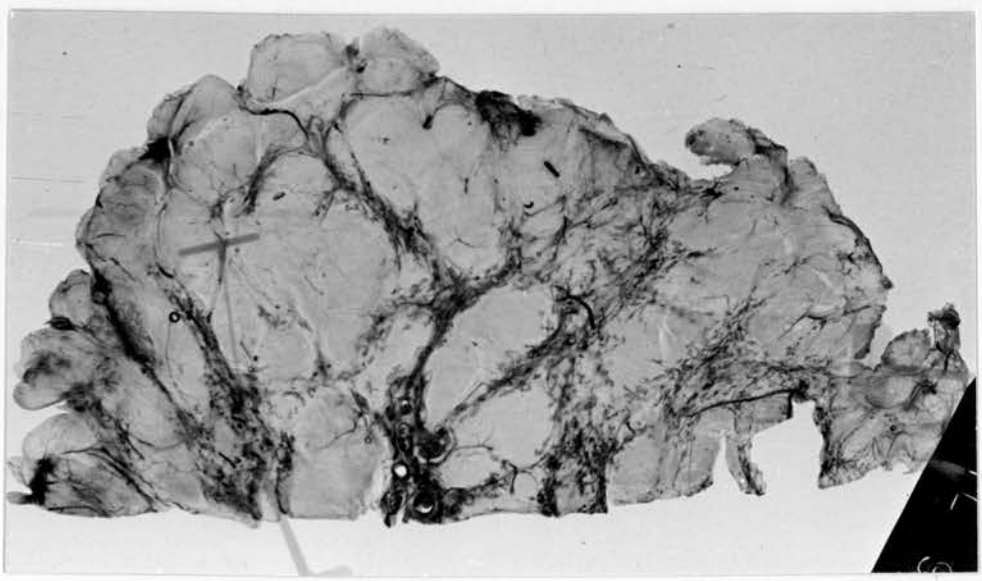
a) Radiograph.

b) Stained (Delafield's haematoxylin) and cleared tissue.

The slice of tissue contains few parenchymatous lobules, but the radiographic appearances are mottled and bear some similarity to Fig. 4.14.



4·15a



4·15b

Fig. 4.16

Coronal slice of breast tissue bisected in a coronal plane (19 years). Mag. x 0.6.

a) Radiograph

b) Stained (Delafield's haematoxylin) and cleared tissue.

The slice of tissue contains numerous parenchymatous lobules, but these are not so evident on the corresponding radiograph as in Figs. 4.13 and 4.14.

4·16a

4·16b

Fig. 4.17

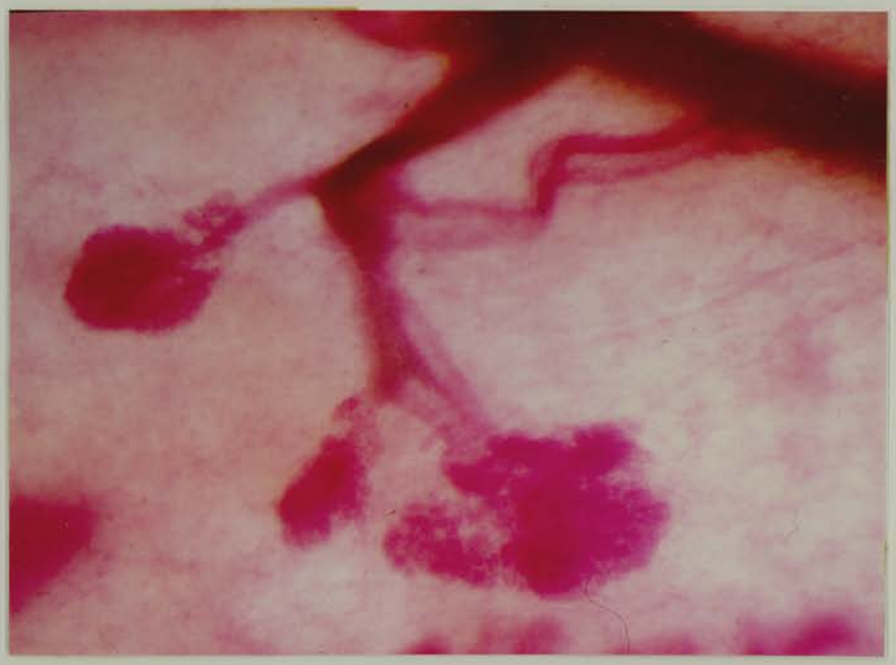
Field of cleared breast tissue - duct injection study (30 years). In the centre of the field an injected duct containing micropaque terminates in solid and intermediate type B lobules.

Delafield's haematoxylin. (Mag. x 40).

Fig. 4.18

Field of cleared breast tissue - duct injection study (30 years). In the centre of the field an injected duct containing micropaque terminates in blind ending ductules or intermediate type A lobules.

Delafield's haematoxylin. (Mag. x 40).



4·17

4·18

DISCUSSION

Examination of morphologically normal subgross lobule types in the breast has indicated that solid lobules are most common in the resting breast of young adults and that the incidence of these solid lobules does not relate to parity (a factor reflecting hormonal activity) or laterality. The incidence of other lobule types increased with age and was also unaffected by either parity or laterality.

It was notable that in the breasts from women 25 years of age and below, although solid lobules were the predominant type, there was a range of lobule appearances. Microcysts, cysts and intermediate type A lobules were found. A hypothesis may be formed that, if as is suggested by observation of subgross appearances and histology, solid lobules are the mature fully developed lobule type in the resting breast, then the breast does not reach its full nulliparous potential for some years after menarche and ovulatory cycles have commenced. Further, it may be the case that, in the large majority of breasts, there are present at any one time a spectrum of lobule types representing focal or widespread phases of development and regression or degeneration. Individual women may have differing potentials for developing solid lobules in the resting state. Clearly a detailed histological study of the normal subgross lobule types is required to define their structure and to confirm whether the intermediate type A and feather-like lobules described in this study are developing or degenerating lobules.

It is unlikely with the use of thick sections that distortion of structures due to processing has affected observations. Wellings *et al.* (1975) state that, in their experience, ducts and lobules that appear normal subgrossly seldom have occult histological or cytological abnormalities when later observed in conventional paraffin embedded sections. Sarnelli *et al.* (1980b) express the opinion that subgross scrutiny of mammary gland tissues usually allows an automatic diagnosis of lesions to be made without the need for histological confirmation. They report that in their study the glandular tree of the breast was more often atrophic than hyperplastic. In further work comparing the effects of ovarian function (i.e. fertility) and the menopause on the type of glandular tree (Sarnelli *et al.*, 1980a), no correlation was found between 'fertility -hyperplasia' and 'atrophy-menopause', but atypical minor lobular changes such as fibroadenomata, apocrine cysts, atrophic lobules, blind terminal ducts and cystic lobules were more frequent in fertile adenosic breasts.

In the present study, attention has not been focused to any great extent on altered parenchymal appearances which, as stated in the introduction, have been studied extensively by other authors. Attention was confined to fibroadenomatous lesions as well as individual cysts and areas of multiple cysts including those showing eosinophilic, i.e. apocrine, change. Fibroadenomata were not commonly observed and were found in breasts of both 19 and 73 years of age which represented the extremes of the age range studied. The incidence of cysts and apocrine cysts which were observed in tissues derived from autopsy as well as mastectomy increased significantly

with age. Although most were recorded in the fourth and fifth decades, the statistical tests reapplied to the results for the breasts from women aged 40 years and above were not significant, indicating that cysts should not be associated predominantly with menopausal women and the elderly. The incidence of cysts with or without apocrine change was unaffected by parity, and areas of tissue containing groups of cysts were more common than both cysts and eosinophilic cysts occurring singly.

Wellings (1980) states that cystic disease is very common both in breasts containing cancer and in those without. Frantz *et al.* (1951) report that 53% of normal breasts are involved with cystic disease and the data of Wellings *et al.* (1975) indicate an even higher percentage. Parks (1959) reports that apocrine cysts are infrequent in early years but, in the fifth decade, numbers of them can be found in most specimens of breast tissue. In the majority of cases they are small and do not present clinically, and there is a great variation in the time sequence of their appearance. Apocrine cysts have been thought of as being proliferative (Cheatle & Cutler, 1931) and degenerative (Dawson, 1943) in character. Eosinophilic changes occur in the involutional phase following lactation and are part of a physiological process (Parks, 1959). The results of the present study support the view that apocrine cysts and microcysts should be considered as physiological constituents of the nulliparous and the resting breast. However, if excessive in number or occurring in quantity at an early age, it may be justified to regard them as abnormal. Parks (1959) states that small cysts are found in all breasts after a certain age and that the variation in the

quantity and quality of duct hyperplasias, lobular hypertrophy and cystic change is so great that the boundaries of normality are broad and hazy and minor degrees of occurrence are probably universal. Similar observations were reported by Kramer & Rush (1973) who found a high incidence of cysts in aged women who died of causes other than cancer of the breast; and Sandison & Walker (1963) found that 9.7% of a large population of women with benign disease of the breast were younger than 21 years of age.

Sloss *et al.* (1957), in a histological study of autopsy breasts from women with no history of disease, report the high incidence of certain of the tissue changes considered to be part of the complex of cystic mastitis. They conclude that the mere qualitative presence of apocrine epithelium, blunt duct adenosis and intraductal epithelial hyperplasias within breasts of women is insufficient to warrant such tissue as being diseased. They were unable to state how common these features are because, to prove that all women display them, would require serial blocking of tissue which is impracticable. This illustrates one advantage of the subgross technique in that large areas of tissue may be reviewed in a relatively short time. Humphrey & Swerdlow (1966) also report a high incidence of cysts and apocrine change in clinically normal breasts examined at autopsy, and stress the need to study once again and re-evaluate breast pathology. Vogel *et al.* (1981) describe subgross morphological changes in the breast in both the epithelial and stromal components related to the menstrual cycle and correlate their results to the ultrastructural findings of Fanger & Ree (1974). They state that,

> ... while changes occurred on a daily basis, these were subtle and subject to inter and intralobular variation in interpretation.

However, only five sections from each specimen were assessed and the experience in the present study of the considerable variation observed within breasts suggests the unreliability of drawing conclusions based on the examination of a limited number of sections.

The use of a duct injection technique followed by observation of stained and cleared slices of tissue to ascertain whether more than one lobule type was associated with a single lactiferous duct system suggested that this was the case (Figs. 4.17 & 4.18). The observation supports the existence of a spectrum of lobule types occurring within individual duct systems rather than the concept of the parenchyma of one entire duct system being at the same stage of development (which may differ from that of another duct system). It may also illustrate a focal nature in hormonal activity in the breast.

In the present study it was observed that, without prior knowledge of the parenchymal structure and abundance within tissue, it was very difficult to predict with confidence the parenchymal composition of a cleared slice of tissue from radiographs. Figures 4.11a and 4.12a illustrate two coronal breast slices from women of 21 and 22 years of age. Figures 4.11b and 4.12b illustrate the respective subgross appearances. The enormous difference in lobular content is immediately evident. Both breasts were firm and fibrous and perhaps reflect the reminder of Dobretsberger (1967) that it is well to remember the individual character of the breast, which not only varies from woman to woman, but also varies in any one

woman depending on factors such as age and hormonal status.

Egan & Mosteller (1977) and Egan & McSweeney (1979) observe that carcinoma in glandular breasts is less frequent and more difficult to detect by mammography compared with the more frequent tumours detected in fatty breasts. The reason for this finding, they suggest, is due to dense fibroglandular tissue delaying the detection of breast cancer by mammography leading to an apparent increase in cancer risk in such breasts. They state that mammography is probably less efficient in detecting the same stage cancers in dense than in fatty breasts and conclude that parenchymal patterns cannot signify which breasts may remain glandular, nor which women may be at risk of cancer.

Jones *et al.* (1982), in a review article on methods of breast imaging, conclude that mammography is currently the most sensitive and specific method of *in vivo* imaging. However, they state the limitations of the technique and, in particular, that results are more reliable when breasts are fatty than when radiographically dense, i.e. fibroglandular. They suggest that a combination of ultrasonic imaging which is not affected adversely by dense glandular tissue and mammography may be diagnostically beneficial. In the present research project it was noted that the less fibrous tissue obscured the parenchyma, the more easily it could be detected by its "mottled" appearance on a radiograph (Fig. 4.14). Kolganova & Zolotarevsky (1981) support this observation by describing the glandular tissue on radiographs as being revealed in 'fine spotted shades'. To achieve optimum results for any slice of tissue, which

should be as thin as possible, several exposures ideally should be made, but in practical terms this is uneconomical. No correlation between radiographic appearances and lobule type or other factors such as age, menstrual cycle status, pill and parity were found.

Hicken *et al.* (1938) state that there is no doubt that the breast can be visualised effectively with contrast substances and it is suggested that, for whole organ radiography, Leborgne (1953) was correct in stating that the only accurate method for distinguishing the parenchyma is by the use of contrast media. Further, excepting the presence of readily diagnosable lesions such as tumours, calcifications or the 'non calcifications' described by Galkin *et al.* (1982), the examination of radiographs of breast tissue slices may be unrewarding since a small increase or decrease in radiographic exposure can produce an altered picture and confuse interpretation.

SUMMARY

A series of 47 whole breasts has been examined and morphologically normal subgross lobule types have been assessed. It was found that the incidence of solid, i.e. mature lobules, decreased significantly with age, whereas that of other lobule types increased significantly with age. Parity and laterality did not affect the results and were not of significance. Duct injection techniques indicated that more than one lobule type was related to individual lactiferous duct systems.

Cysts and apocrine cysts occurred in breasts throughout the age range of 19-73 years and their incidence increased significantly with age. Parity and laterality did not influence the results. Fibroadenomata were not commonly observed and were not restricted to the breasts of younger women.

Observation of radiographs of breast slices suggested that subgross lobule type and parenchymal abundance cannot be predicted accurately from radiographic appearances alone.

CHAPTER 5

MUCOPOLYSACCHARIDES IN THE BREAST

INTRODUCTION

Premenstrual tension, first described by Frank (1931), has many symptoms, including fatigue, depression, irritability, swelling of the breasts, thirst, abdominal bloating and weight gain a few days before each period. The physical signs of premenstrual tension are oedema, which is best noted at the fingers and ankles, and a temporary gain of weight (Israel, 1967) but not all the authors believe that the severity of the symptoms is directly linked to the weight gained (Bruce & Russell, 1962). During the normal menstrual cycle there are overall changes in the amount of fluid retained in body tissues but the total change in weight in most women is no more than 1 kg (Parboosingh, Doig & Michie, 1973). Some authors believe that psychogenic factors are responsible for this premenstrual syndrome and that environmental stresses may play a role. However, both excess progesterone (Givens *et al.*, 1975) and altered oestrogen and progesterone levels (Israel, 1938; Morton *et al.*, 1953) have been suggested as causing the syndrome from which anovulatory women do not appear to suffer (Scommegna, Vorys & Givens, 1980).

Landau *et al.* (1955) suggest that progesterone withdrawal or a decrease in progesterone and oestrogen late in the luteal phase of the cycle may be associated with alteration of the renin-angiotensin-aldosterone system, thereby inducing water retention and an increase in extracellular fluid. Thorn, Nelson & Thorn (1938) demonstrated increased salt and water retention premenstrually with increased

renal excretion of these substances during menses. However, although this could explain oedema, it could not account for the rest of the symptoms of the premenstrual syndrome which are not found in other conditions associated with water retention, such as cardiac failure.

Most normal women experience some changes in their breasts during the menstrual cycle and several workers have recorded changes occurring in the premenstrual volume of the whole breast. Using a water displacement method, Milligan, Drife & Short (1975) found increased breast volumes in the second half of both normal and contraceptive controlled cycles. Geschickter (1945) also recorded raised breast volumes in the luteal phase by water displacement. Taylor (1936) maintained that premenstrual hyperaemia in the breast was "obvious" and noted that most women experience swelling and heaviness in their breasts a few days before each period. Pickles (1953) believed that increased volumes of the breast might be due to a water retention following increased mammary blood flow in the luteal phase of the cycle. Haagensen (1971) also presumed that breast enlargement at this time of the cycle must be due to blood or lymph engorgement; or else to "changes in the extracellular fluid tension which cannot be demonstrated with ordinary microscope techniques."

Vascular changes in the breasts have been studied by Isard & Shilo (1968) who performed weekly thermograms on 10 healthy volunteers aged 20-43 years. In 5 women there was increased vascularity in the week prior to menstruation, although for the

whole sample correlation with the menstrual cycle was poor. Parry, Freundlich & Wallace (1972) studied 9 healthy women using thermography and also found no cyclical changes. They suggest that cyclical changes reported by previous investigations were probably found because changes in basal body temperature occurring in the second half of the cycle had not been compensated for.

As a result of planimetric measurements taken from radiographs of breasts before, during and after menstruation, Reimann & Seabold (1933) found that the size of the breast tissue varied with the menstrual cycle and was at its greatest 7-10 days before the onset of flow. Ingleby (1949), by studying plaster cast models, also recorded increased premenstrual breast volumes but stated that, although the volume of the breast appears to change, the methods available to measure this are imperfect and changes in elasticity and density may give rise to misleading results. Hamilton & Rankin (1975) suggest that perhaps the difficulty in achieving reproducible measurements accounts for the paucity of information on the nature of changes in breast volume. In studies on breast pain, Preece *et al.* (1974) were unable to find any changes in total body water during the menstrual cycle.

At a histological level, several theories have been proposed to account for menstrual cycle changes occurring in the parenchymal tissue of the human breast. Changes in total breast volume have been largely attributed to cyclical growth and regression of lobules, cyclical production of secretions, and cyclical oedema of stromal elements. The difficulties involved in assessing histological changes are considerable as indicated by Pallot (1935)

who stated: "If a breast presents uniform appearances throughout, that breast is not normal."

In the 1920s a number of workers described cyclical changes in the breast stroma and parenchyma. Rosenburg (1922, 1923) believed that lobules formed just prior to menstruation and afterwards involuted and disappeared. Berberich & Jaffe (1924), Polano (1924), Ernst (1925), Loeschke (1925), Sebening (1925), Luchsinger y Centeno (1927) and Foote & Stewart (1945a) are amongst other authors who supported a similar theory but with some variations, most important of which was disagreement with the complete involution of lobules which Rosenburg described in the intermenstrual phase. Several of the authors believed instead that parenchymal tissue undergoes development and regression in a focal, rather than diffuse, pattern throughout the breast during the menstrual cycle. Dieckmann (1925) described fullness and distention of the breasts in the premenstrual phase on the basis of lobular oedema, and catalogued different histological changes occurring in the parenchyma for each phase of the menstrual cycle. Moszkowicz (1926) believed that both epithelial and, more markedly, stromal alterations were involved in cyclical changes in the breast. He observed premenstrual oedema of connective tissue, cellular detritus in ducts and acini and transitory secretion in some glandular areas. According to Ingleby (1932):

> The amount of new growth taking place in the breast at each sexual cycle is astonishing ... a point to be borne in mind is that the connective tissue of the breast proliferates and degenerates in inverse ratio to the epithelium.

In contrast, Haagensen (1971), Wilson (1976) and Drife (1982) were

unable to attribute either cyclical changes occurring in the breast or fullness of the breasts associated with premenstrual tension to lobular oedema.

Ingleby & Gershon-Cohen (1960) divided menstrual cycle changes in the breast into proliferative and secretory phases. Their description bears a close resemblance to the cyclical changes described in the endometrium (Hitschmann & Adler, 1908). Premenstrually, in the ductular cells, they observed protein secretion on the cell surfaces which discharged into the lumina. Papanicolau *et al.* (1958), in a study of exfoliative cytology of nipple secretion, found that, among women aged 20-39, secretion could be aspirated from some 23% of women in the second week of the cycle and from 55% in the 4th week. Details regarding parity were not given and a cyclical change was not observed in women over 40 years. Petrakis *et al.* (1975) state that secretions could be aspirated from approximately 75% of non-lactating premenstrual women. Parity and menstrual cycle did not affect the result but the menopause did, causing the percentage to fall to 60%. Bonser *et al.* (1961) express no doubts that the changes which produce fullness of the breasts prior to menses are due to variation in secretions and their reabsorption with changing hormonal conditions. They relate their work to that of Ozzello & Speer (1958) who describe an increase in acid mucopolysaccharide levels occurring in the intra-lobular stroma of the breast parenchyma in the luteal phase of the menstrual cycle. Bonser *et al.* (1961) propose that the major substance contributing to this reaction is hyaluronic acid and they suggest that the variation in the acid mucopolysaccharide content of

the breast stroma is dependent upon oestrogenic stimulation. Agreeing with Bonser *et al.* (1961) and Ingleby & Gershon-Cohen (1960), they also report finding a cyclical occurrence of secretions in the breast. However, their results are based on a subjective assessment of both normal and pathological breast biopsy specimens and their findings in relation to cyclical stromal variations do not appear to have been confirmed either by themselves or by other workers.

Metachromasia is a staining reaction exhibited by various tissue components towards certain basic dyes, whereby they are stained a different colour to that of the dye employed. Whereas several authors have described metachromasia and positive alcian blue staining occurring in the intralobular stroma of normal breast tissue (Wislocki, Bunting & Dempsey, 1947; Ozzello & Speer, 1958; Meriggi, Azzini & Chiesa, 1964; Pozzi, Meriggi & Dagrada, 1964), few have attempted to characterise the staining reactions further. Ihnen & Perez-Tamayo (1953) relate the metachromatic effect to the collagenous content of lobules. They define cellular, fibrillary and collagenous stroma and state that the former two "types" of lobule are associated with metachromasia in the premenstrual phase of the cycle. They believe that the substances which cause the histochemical reaction are chiefly hyaluronic acid and chondroitin sulphate. Mancini *et al.* (1951) also relate metachromasia to the presence or absence of collagen fibres. Huseby & Thomas (1954) correlate changes in the collagen content of the intralobular stroma, i.e. "mantle" tissue of Berka (1911), to age rather than to the menstrual cycle. According to Kuru (1909) and Sylvén (1938) the

intralobular stroma is not metachromatic. Other studies of mucin histochemistry in the breast have concentrated on pathological aspects of the organ (Grishman, 1952; Olivi & Barbieri, 1952; Leuschner, 1969; Cooper, 1974; Tavassoli & Norris, 1980).

Connective tissues contain many compounds made by covalent combinations of proteins and polysaccharides, with a spectrum of composition ranging from mostly proteins to mostly polysaccharides. At one end of the spectrum are the proteoglycans in which the protein components, being in the order of 5%, are a very minor part of the total mass. These very large polyanions bind water and cations and thereby form the ground substance in the extracellular matrix of connective tissues, contributing up to 30% of the dry weight of the tissue (White *et al.*, 1978). They have long heteropolysaccharide chains covalently attached to a protein core much like the bristles on a brush.

The polysaccharide groups of the proteoglycans were formerly termed the mucopolysaccharides, a name first used by Stacey & Barker (1962), but the name glycosaminoglycan is now preferred since all contain derivatives of either glucosamine or galactosamine. At least one of the sugars has a negatively charged carbohydrate or sulphate group (Stryer, 1981). Six major groups of glycosaminoglycan are recognised:

Hyaluronic acid

Chondroitin-4-sulphate (type A)

Chondroitin-6-sulphate (type C)

Dermatan sulphate (chondroitin sulphate type B)

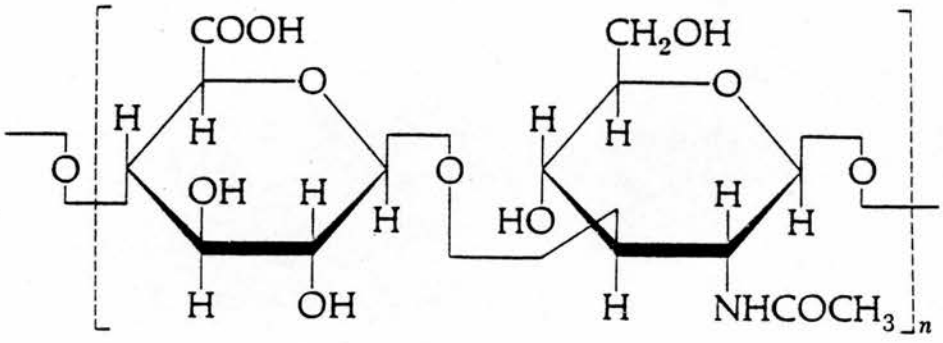
Keratan sulphate (types I and II)

Heparan sulphate and heparin

Although in biochemical terms glycosaminoglycans is now the correct name for the mucopolysaccharides, in this study the latter term will be used to avoid confusion with the literature, much of which was written prior to the new terminology.

Mucopolysaccharides have complex staining reactions. When attempting to identify a given mucopolysaccharide in a tissue, there is the difficulty not only that a mixture of types may occur but also that there are varying degrees of binding with other tissue components. Mucopolysaccharides may be divided into two broad categories: neutral and acidic. Neutral mucopolysaccharides consist of hexosamine and hexose units and do not have free acidic groups. On staining they are alcian blue (AB) negative and periodic acid-Schiff (PAS) positive. Acid mucopolysaccharides consist of hexosamine units which may be associated with glucuronic, iduronic or sialic acids. Sulphate radicals may also be present. Acid mucopolysaccharides may be very broadly divided on the basis of their composition into strongly sulphated, weakly sulphated and non-sulphated acids. They are considered to be largely PAS negative and may be differentiated by AB positivity at varying pH (Cook, 1982).

Hyaluronic acid is a non-sulphated uronic acid containing mucopolysaccharide which was first isolated by Meyer & Palmer (1934). It contains only approximately 1-2% protein by weight and has a long heteropolysaccharide chain composed of some 5,000 carbohydrate residues with a molecular weight of 1-3 million. The chain has no sulphate groups and contains alternating residues of D-glucuronate and N-acetyl-D-glucosamine. Its structure is given below:-



Repeating unit of hyaluronic acid

The molecular chain of hyaluronic acid coils in a "relatively random" manner and there is entanglement of the macromolecular chains even at low concentrations. The volume occupied by a mucopolysaccharide molecule is called its "domain". The domain of hyaluronic acid is largely filled with solvent water. One gram of hyaluronic acid in excess water will exclude other large molecules from approximately 1 litre of space and will become 1,000 times the volume of the anhydrous molecule (McGilvery, 1979). In concentrated solutions the chains collapse and interpenetrate accounting for the high viscosity of such solutions. The potential magnitude of the domains may be appreciated by the fact that the domain of a hyaluronic acid molecule is 75,000 times the volume occupied by three rigid densely packed tropocollagen roads which together would have the same molecular weight (White *et al.*, 1978). It was concluded by Hvidberg & Jensen (1959) that the size of the hyaluronic acid molecule

is an important factor in the water binding mechanism of connective tissue.

Hyaluronic acid does not form a gel since it does not cross link, and holds rather than binds water in the tissues. The acid may, however, be spoken of as a gel since it exhibits some of the relevant characteristics, especially restriction of other macromolecules by a molecular sieving action (Schiff, 1966). Smaller molecules or ions have access, but larger molecules such as serum albumin may be excluded. The higher the molecular weight of hyaluronic acid, the greater is the restriction and sedimentation as well as the lower the diffusion rate. The molecular diameter or size of the moving particle is the most important factor in the response to molecular sieving. In a series of sophisticated experiments, Laurent & Ogston (1963), Laurent *et al.* (1963), Laurent (1964) and Laurent & Killander (1964) determined some of the physical measurements of the transport of globular proteins through hyaluronic acid solutions.

Hyaluronic acid occurs in small quantities in many sites, but is principally associated with synovium, skin, umbilical cord, bone and cartilage. The chondroitin sulphates which were divided into three types by Meyer & Rapport (1951) are also found in several tissues. Type A is found in cartilage only. Type B occurs in heart valves, aorta and skin. Type C is found in heart valves, skin, cartilage and umbilical cord. The chondroitin sulphates and hyaluronic acid therefore are often found occurring simultaneously and are isolated from one another with difficulty.

In this study it was decided to investigate quantitatively the presence of acid mucopolysaccharides in the intralobular stroma of the normal breast. The relationship between a hyaluronic acid component of acid mucopolysaccharides occurring in the intralobular stroma and cyclical hormonal variations was examined. Further, the presence and the relationship to the menstrual cycle of both intracellular granules within ductular cells and intraluminal secretions in ductules were investigated.

Oedema is a term which is often used in an unspecific manner and must be defined for the purposes of this study. It is the name used to describe an excessive accumulation of serous fluid in the extracellular spaces of tissues. There are many different 'types' of oedema described in medicine, e.g. the oedema associated with premenstrual tension is not the same as the idiopathic oedema induced by secondary aldosteronism in that it is not affected by gravity. In the literature the "oedema" which is associated with hyaluronic acid accumulation in the ground substance implies a simple retention of water, since hyaluronic acid holds rather than binds water within its molecules. The water retention associated with a premenstrual accumulation of hyaluronic acid in the ground substance of intralobular breast stroma may therefore be likened to the physiological oedema described in other organs and tissues during the premenstrual phase of the menstrual cycle.

MATERIALS

Breast biopsies were used to assess quantitatively and qualitatively the presence of mucopolysaccharides. Whole breast slices were used to observe the distribution of mucopolysaccharides in larger areas of tissue.

Normal breast tissue was selected from 43 breast biopsies obtained from the Well Woman Breast Screening Clinic at Longmore Hospital, Edinburgh. The age range of the patients was from 19 to 53 years, and, of these, 50% were below 30 years of age. Table 34 in Appendix C lists the specimens and provides details regarding the age of each patient, laterality, the histological diagnosis and size of each biopsy obtained, the number of blocks examined, as well as the stage of the menstrual cycle at the time of surgery. For each patient the exact day of the menstrual cycle at the time of surgery was calculated by obtaining the date of onset of menses prior to surgery together with the date of onset of the subsequent cycle.

Biopsies represented as many days of the menstrual cycle as possible and only days 8, 14, 18 and 19 were not represented. Normal tissue was obtained from the edge of a lesion at the time of biopsy and only tissues adjacent to lesions which were diagnosed histologically as benign or normal were included.

Controls

Two biopsies histologically diagnosed as breast fibroadenomata of pericanalicular type were used as controls in the experiment.

This material was routinely processed paraffin embedded tissue and had not been subjected to the subgross staining and clearing technique.

METHODS

STAINING AND CLEARING OF TISSUE

The breast biopsies were fixed in 4% formaldehyde in phosphate buffer fixative (Carson *et al.*, 1973). The tissue was cut into approximately 1 - 2 mm thick slices using a skin graft knife blade. The slices were placed in wire mesh Histokinette baskets and processed using the following schedule on a Histokinette automatic tissue processor (Reichert-Jung, Slough, Bucks.):-

1. Wash in running tap water 15 minutes.
2. Stain in Delafield's haematoxylin 1 hour.
3. Wash in running tap water 1 hour.
4. Decolourise in acid ethanol (2% hydrochloric acid/ethanol) 1 hour.
5. Differentiate by washing in running tap water 30 minutes.
6. Dehydrate in 95% ethanol 3 hours.
7. Dehydrate in 99% ethanol 3 hours.
8. Dehydrate in 99% ethanol 3 hours.
9. Dehydrate in 100% ethanol 4 hours.
10. Dehydrate in 100% ethanol 4 hours.
11. Clear in methyl salicylate 2 hours.

To allow washing in running water, the Histokinette beakers were adapted with an inlet and outlet connected to the water system. The cleared slices were removed from the Histokinette and placed in transparent bags (Kapak Corporation, Bloomington, USA) which were heat sealed.

This automated technique for the rapid processing of breast tissue for subgross examination has been published (Manton, Ferguson & Anderson, 1981). It has been found that the technique does not affect subsequent histological staining. Haematoxylin and eosin, toluidine blue and alcian blue/periodic acid-Schiff stains have been successfully carried out on the tissue.

SELECTION OF TISSUE FOR MICROTOMY

Following the staining and clearing procedure, blocks of tissue from the 43 specimens were examined using the stereomicroscope. Blocks not displaying parenchyma were discarded. Where possible, blocks containing only one lobule type (see chapter 4) determined by the subgross appearances were selected. From biopsies composed of only one subgross type of lobule, one block of tissue was selected for microtomy. From cases where slices of tissue from the same biopsy varied in the lobule types they displayed, more than one block was selected to represent the parenchymal composition of the case. In all, 70 blocks from the 43 cases were processed for microtomy and histology. Each was photographed prior to processing in order to provide a record of the lobule types occurring in the

tissue. Details of photographic equipment and use of film are provided in Appendix A, XIV.

PROCESSING FOR MICROTOMY

The embedding cycle for both unstained tissues and tissues subjected to staining and clearing techniques is given in detail in Appendix A, XI.

MICROTOMY AND MOUNTING OF SECTIONS

Six serial sections at 3 μm were obtained from all the blocks, including the two controls, using a Leitz 1212 Minot-type Rotary Microtome (Leitz Ltd., Luton, England). The sections were floated out on tap water (45^{0}C). After mounting with Coverbond T.M. (Harleco, The American Hospital Supply Corporation (UK), Berks.) on 7.5 cm x 2.5 cm glass slides, the sections were dried at 70^{0}C for 30 minutes.

SELECTION OF STAINING METHODS

The following stains have been used, the staining schedules and histochemical methods for which are reproduced in Appendix A, XI and XII.

(a) Alcian blue (AB)

Alcian blue is a copper phthalocyanin dye (Haddock, 1947) and was first proposed as a mucin stain by Steedman (1950).

For general staining of acid mucins, a 1% solution in 3% acetic acid may be used to give a pH of 2.5 (Lev & Spicer, 1964). At this pH, both sialomucins and sulphomucins are demonstrated by their alcianophilia. Acid mucins may be separated in a critical manner by varying the pH of the staining solution, e.g. pH 0.5 - 1 is generally considered to stain only sulphate esters.

The duration of staining with AB is not critical, provided that a standard time is adopted (Cook, 1972), as AB stains rapidly to a maximum degree which is not influenced by further exposure to the stain.

Cook (1972) states that solutions tend to deteriorate with age and fresh solutions should therefore be used. Lamb (1968) and Going (1977) found that they obtained variations in AB staining intensity between batches of control slides, and they were unable to attribute this to alterations in technique. As a result, Going (1977) suggested that the AB dye itself may suffer alteration to its staining properties with time.

Lamb (1968), Cook (1972)and Going (1977) agree that the type of fixative used with mucins is not very critical. Post mortem alterations in mucin histochemistry are not significant if periods up to 48 hours are involved (Lamb, 1968). Allison (1973), in an investigation of the effects of 22 immersion type fixatives on the subsequent demonstration of mucopolysaccharides in the dog

submandibular salivary gland, concludes that no fixative performed better than 10% formal saline.

Alcian blue is strongly retained by celloidin and thus the use of celloidin sections should be avoided.

(b) The periodic acid-Schiff reaction (PAS)

In practice, neutral mucins invariably give positive results with the periodic acid-Schiff (PAS) reaction, due, it is thought, to the presence of a hexose component.

Acid mucins may, or may not, be PAS positive (Cook, 1972). Although there is some controversy surrounding the PAS-positivity of acid mucins, the connective tissue mucins such as hyaluronic acid are usually negative (Quintarelli *et al.*, 1960).

(c) Combined alcian blue/periodic acid-Schiff (AB/PAS) method

If an AB stain, at pH 2.5, is combined with the PAS reaction, neutral mucins will stain first prior to exposure to the AB stain. However, some mucins will be both AB positive and PAS positive and will stain in intermediate colours between the turquoise of pure AB and the magenta of the pure basic fuchsin in the Schiff's reagent. The combined AB/PAS method (Mowry, 1956) is thus a differential method for acid and neutral mucins.

For reproducible results, standard times and temperatures of staining are essential. They need to be determined by experiment and results obtained with one batch of Schiff's reagent must not be applied to other batches.

The AB, pH 2.5/PAS staining schedule used throughout this study was determined as a result of extensive fixation and staining trials (Appendix A, XIII).

(d) Diastase digestion

Digestion by diastase excludes the presence of non mucin carbohydrate such as glycogen. Starch also is digested but more slowly. It is thought that diastase hydrolyses glycogen and starch to maltose which is then dissolved out (Bernfield, 1951). Traditionally, the source of diastase is saliva but this is rather uncontrollable and therefore diastase extracted from malt is preferred.

(e) Hyaluronidase digestion

Mannozzi-Torini (1942) first introduced the use of this enzyme which has become important for the demonstration of hyaluronic acid. The source of hyaluronidase most commonly used is the bovine testis. This will digest, in addition to hyaluronic acid, chondroitin sulphates A and C.

Bacterial hyaluronidase is more selective in its action and Bunting (1950) demonstrated that only hyaluronic acid is digested. However, it is extremely difficult and expensive to obtain and was unavailable for use in this study. Testicular hyaluronidase, supplemented with a toluidine blue staining method was therefore used to distinguish hyaluronic acid from the chondroitin sulphates A and C.

(f) Toluidine blue

This stain was employed to demonstrate the presence of mast cells and also, at a pH of 2.0, following hyaluronidase digestion (Szirmai & Balazs, 1958), to aid the differentiation of hyaluronic acid from chondroitin sulphates A and C.

(g) Van Gieson's stain

This staining method was used to demonstrate collagen occurring in the intralobular stroma in order to examine the relationship between the intensity of the AB staining reaction and the quantity of collagen present.

The techniques for all the staining methods listed above are described in Appendix A, XII.

PROCESSING OF WHOLE BREAST SLICES

A stained and cleared whole breast slice from each of 10 breasts was selected. The slices were embedded in 6 stages: the first 3 in chloroform for 5 hours each and the last 3 in Polywax (Difco Labs., East Molesey, Surrey) for 4 hours each. Two sections at 7 µm were cut from each block using a tetrander microtome (Reichert-Jung UK, Slough, Bucks.). One section was stained with H & E in the normal manner and the other with AB/PAS as described. Processing of the tissue was performed at St Luke's Hospital, Guildford, Surrey.

Fig. 5.1

Histological preparation of breast tissue (32 years) illustrating +++ alcian blue staining of the intralobular stroma.

Stained AB/PAS. Mag. x 120.

Fig. 5.2

Histological preparation of breast tissue (30 years) illustrating ++ alcian blue staining of the intralobular stroma.

Stained AB/PAS. Mag. x 120.

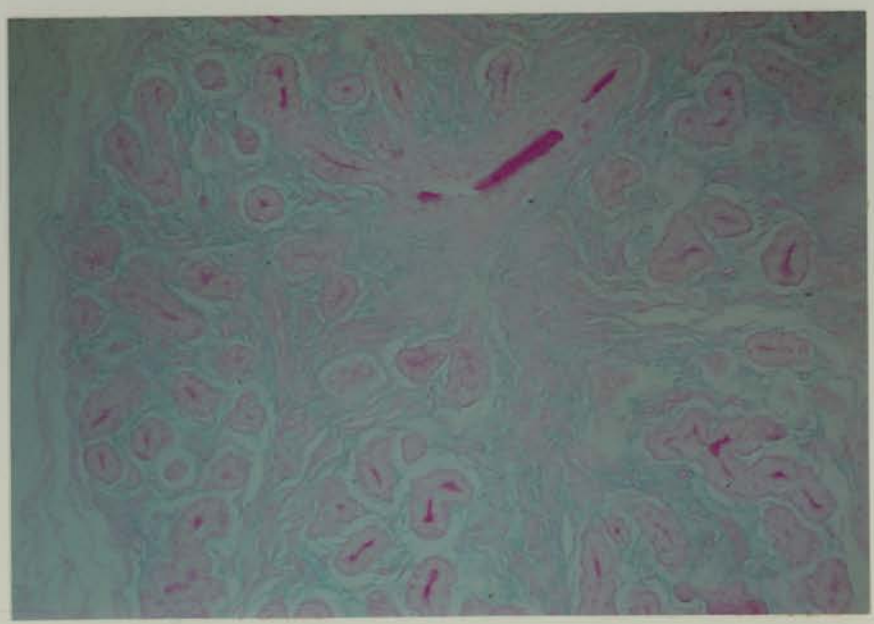
Fig. 5.3

Histological preparation of breast tissue (24 years) illustrating + alcian blue staining of the intralobular stroma.

Stained AB/PAS. Mag. x 120.

5·1

5·2



5·3

HISTOLOGICAL EXAMINATION OF SECTIONS

A proforma was constructed to facilitate the recording of data. This is reproduced in Appendix C, Table 35. The following features of each AB/PAS section were assessed using an Ortholux II light microscope (E. Leitz Ltd., Luton, England).

1. Number of lobules

All the lobules on each section were assessed, with the exception of the qualitative assessment of intracellular granules when up to a maximum of 50 lobules on any one section were examined.

2. For each lobule

(a) Number of ductules

The number of ductules in each lobule was recorded.

(b) Stroma

The AB positivity in the intralobular stroma was recorded as:-

0	no staining reaction
+	a mild staining reaction
++	a moderate staining reaction
+++	a heavy staining reaction

A control slide of fibroadenomatous breast tissue was stained with AB only.

A "heavy" staining reaction in the intralobular stroma was taken to be equivalent to the AB staining reaction on control AB/PAS slides obtained from 2 cases of breast fibroadenomata.

The staining intensity attributed to the "mild" and "moderate" staining reactions was selected by two observers who separately scanned a number of slides at random prior to the main experimental assessment of the 70 sections. An example of each of the staining reactions was photographed to provide colour transparencies to use as controls during assessment. Figures 5.1-5.3 are prints which represent the best reproduction that was obtained from the transparencies used.

The sections were examined using the light microscope at x 10 and x 25 magnifications.

(c) Secretions

Presence of secretions within the lumen:

0 no secretions in a lobule

\+ up to one-third of the ductules in a lobule

++ one to two-thirds of the ductules in a lobule

+++ over two-thirds of the ductules in a lobule

Colour of the secretions: on AB/PAS sections 5 colours of staining were recognised:

(i) Pure magenta (R), the colour of the Schiff's reagent staining alone.

(ii) Pure turquoise blue (B), the colour of the AB staining alone.

The staining intensity and colours obtained when AB and Schiff both react on a single glycoprotein depend on the extent and balance of acid residues and aldehyde side chains reacting to the two stains. The colours that result are shades of purple representing a mix of AB and Schiff staining reactivity.

These colours (iii), (iv) and (v) are defined as follows:

(iii) Schiff staining predominating over AB staining (R/B).

(iv) AB staining predominating over Schiff staining (B/R).

(v) Where both AB and Schiff stain heavily at the same location, a dark navy blue (N) staining reaction results.

The presence of any of the colours occurring in the secretions of the ductules in a lobule was recorded. The assessment was not designed to determine the predominant colour of secretions occurring in a lobule but was, rather, intended to record all the colours which were observed so that any cyclical changes occurring in the secretions in relation to the menstrual cycle could be detected.

As it was found by reviewing a random selection of sections that none of the secretions showed a pure turquoise blue (B) staining reaction, and only very rarely was an AB predominating over Schiff (B/R) reaction observed, the secretions were either R, R/B or N. These colours are easily recognised and therefore colour standards were not employed.

Diastase digestion, followed by AB/PAS staining was performed on 20 blocks from 16 biopsies showing +++ as well as differing types of staining reaction, to ascertain whether the secretions had any glycogen component.

(d) Intracellular granules

Evaluation of intracellular granules occurring within ductular cells was performed as follows:

Presence of granules. Intracellular granules occurring within the ductular cells in a lobule were assessed according to:-

0 granules not present in the ductules of a lobule

\+ granules occurring within the cells of up to one-third of the ductules in a lobule

++ granules occurring within the cells of one to two-thirds of the ductules in a lobule

+++ granules occurring within the cells of over two-thirds of the ductules in a lobule

The size and number of granules occurring within any one ductule were not taken into account. Presence or absence of granules within cells was the object of the examination.

The sections were observed at x 25 and x 40 magnifications using the light microscope. In order to eliminate a potential source of confusion between the identification of intracellular granules and the occurrence of apocrine metaplasia, H & E sections were examined for the presence of apocrine cells prior to the assessment of the AB/PAS sections. Also, sections with 15 and over lobules assessed as +++ reactions for granules were stained with toluidine blue to demonstrate the presence of mast cells.

Size and colour of the granules. The diameters of the intracellular granules were calculated using a 1 cm graticule and a 0.01 mm micrometer slide, using x 40 and x 100 (oil immersion) magnifications. The colour of staining of the granules with AB/PAS was recorded.

Qualitative assessment of the granules. The estimate of the presence of the granules within ductular cells was designed to ascertain

whether granules were observed within ductular cells during all phases of the menstrual cycle. Such an estimate, however, is not qualitative, and therefore does not take into consideration factors such as the differing locations of granules within the cell, or their composition.

A second assessment of the AB/PAS sections for all the cases was therefore performed in an attempt to categorise the presence of the granules in a more qualitative manner. It was assumed that if, as Ozzello & Speer (1958) suggest, granular material discharges into the lumen premenstrually, then "secretory" granules about to discharge will be found in a subluminal position.

All the AB/PAS sections were re-examined and the positioning of granules intracellularly was recorded by assessing the presence of:

i) Large intracellular granules occurring in a subluminal position.

ii) High concentrations of small intracellular granules occurring in a subluminal position.

iii) A combination of i) and ii) occurring simultaneously.

iv) Large intracellular granules occurring at sites other than subluminal.

Diastase digestion was performed on 20 sections which displayed large quantities of secretion and/or granules in subluminal sites within ductules in order to test for the presence of glycogen as a significant component of their structure.

RESULTS

CYCLICAL REGRESSION ANALYSES

Variation in the mucopolysaccharide element throughout the menstrual cycle was assessed by fitting sinusoidal curves with a 28 day cycle to a transformation of these values. This was achieved by performing a linear regression of measures of acid and neutral mucopolysaccharides against the sine and cosine of the phase angle during the menstrual cycle (varying from 0^0 to 360^0 from day 1 to day 28). The regression tests of significance were used to establish whether or not the cyclical variation was more than a chance effect.

The computer programme used for the analyses was a multiple linear regression (programme PIR from the BMDP biomedical computer programme P series; Dixon *et al.*, 1981).

Analyses were performed on the first section of the first block of tissue assessed for each patient. Graphs to illustrate the results were obtained following the regression analyses. All raw data and scores calculated for the assessment of the intralobular stroma, luminal secretions and intracellular granules within ductular cells are presented in Appendix C, Tables 36-41.

INTRALOBULAR STROMA

The level of acid mucopolysaccharide occurrence in the intralobular stroma of lobules has been expressed in terms of a score awarded according to the strength of AB staining observed (STR score).

STR Score

The stromal score for lobules displaying AB staining (STR score) was calculated as follows:-

For each section,

if NL = total number of lobules

and N1 = number of lobules scoring + staining

N2 = number of lobules scoring ++ staining

N3 = number of lobules scoring +++ staining

$$\text{then STR score} = \frac{(N1 \times 1) + (N2 \times 2) + (N3 \times 3)}{NL}$$

Thus, the greater the number of lobules with +++ staining, the higher the score obtained (which has a potential range of 0-3). The scores for all the cases were put through a regression analysis as described and the results plotted in graphical form (Fig. 5.4). On the graph, the predicted curve showing cyclical variation is illustrated. The result is significant ($p < 0.05$) and peaks at approximately day 24.

STR score for +++ lobules only

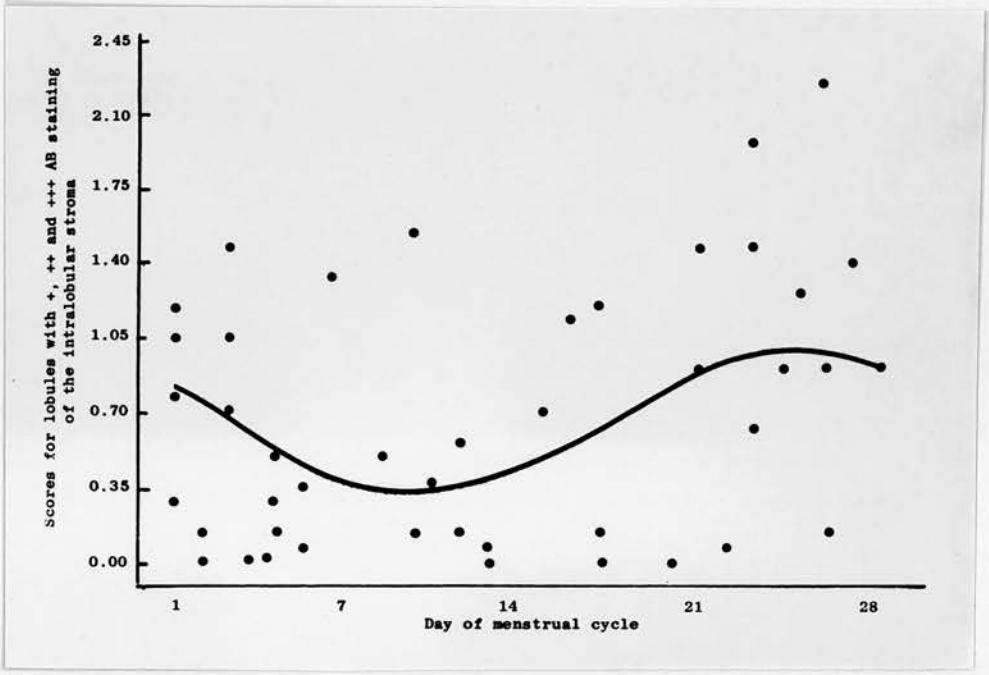
The +++ component of the STR score was examined for its contribution to the overall cyclical variation. The score for the

Fig. 5.4

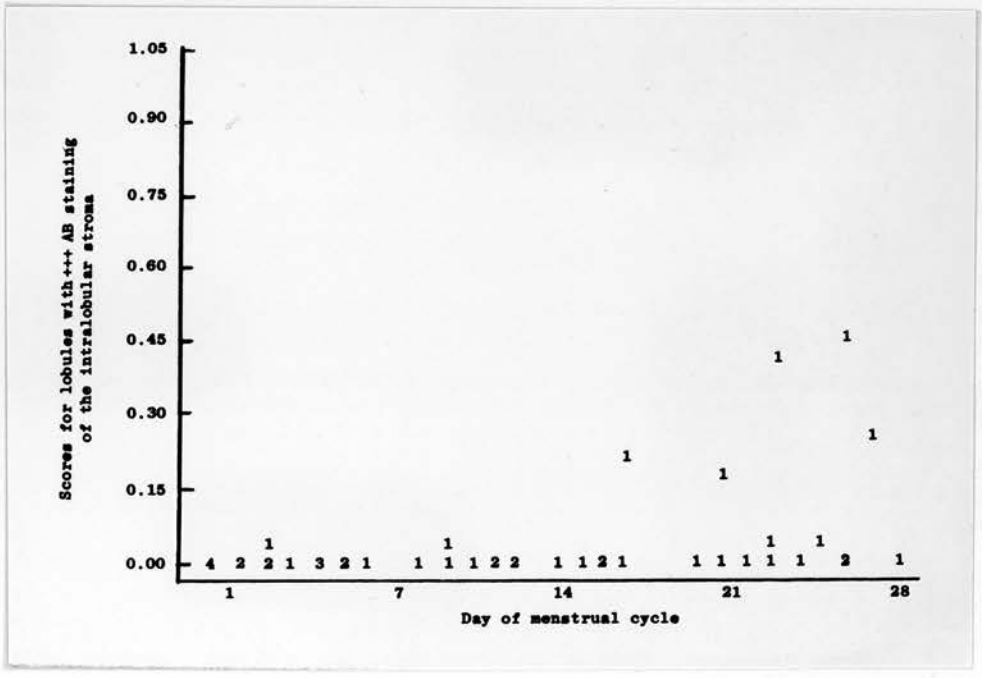
Variation in combined stromal scores with day of menstrual cycle for 43 breast biopsies. Cyclical variation which was significant ($p < 0.05$), is shown as a curve which peaks at approximately day 24.

Fig. 5.5

Variation in +++ stromal scores with day of cycle for 43 breast biopsies. The figures plotted represent numbers of cases.


Scores for lobules with +, ++ and +++ AB staining of the intralobular stroma
2.45
2.10
1.75
1.40
1.05
0.70
0.35
0.00
1
7
14
21
28
Day of menstrual cycle

5.4


Scores for lobules with+++ AB staining of the intralobular stroma
1.05
0.90
0.75
0.60
0.45
0.30
0.15
0.00
1
7
14
21
28
Day of menstrual cycle

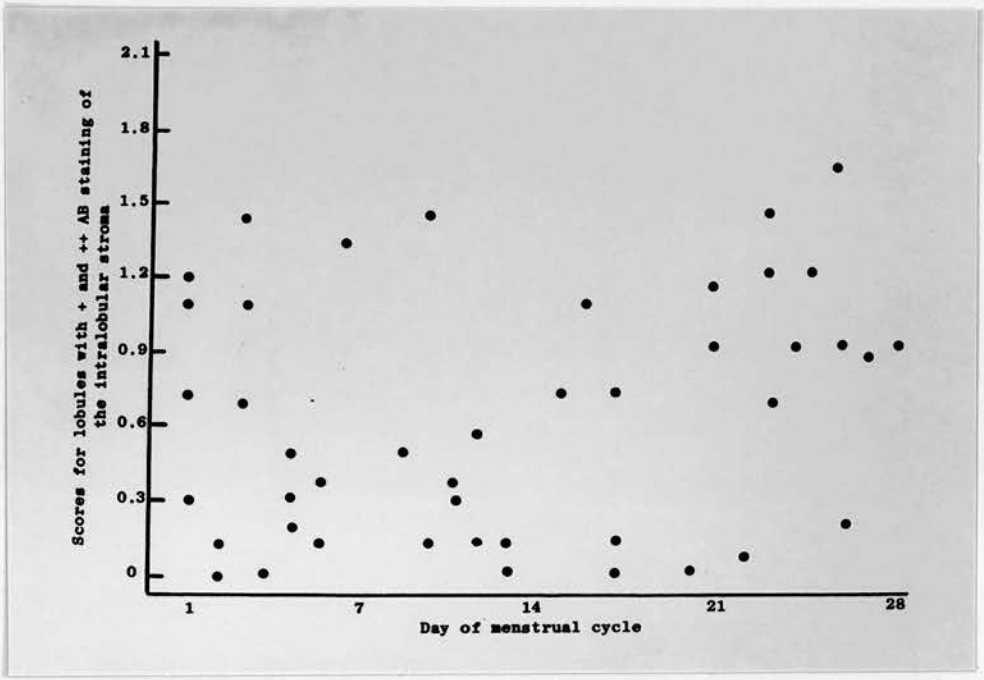
5.5

Fig. 5.6

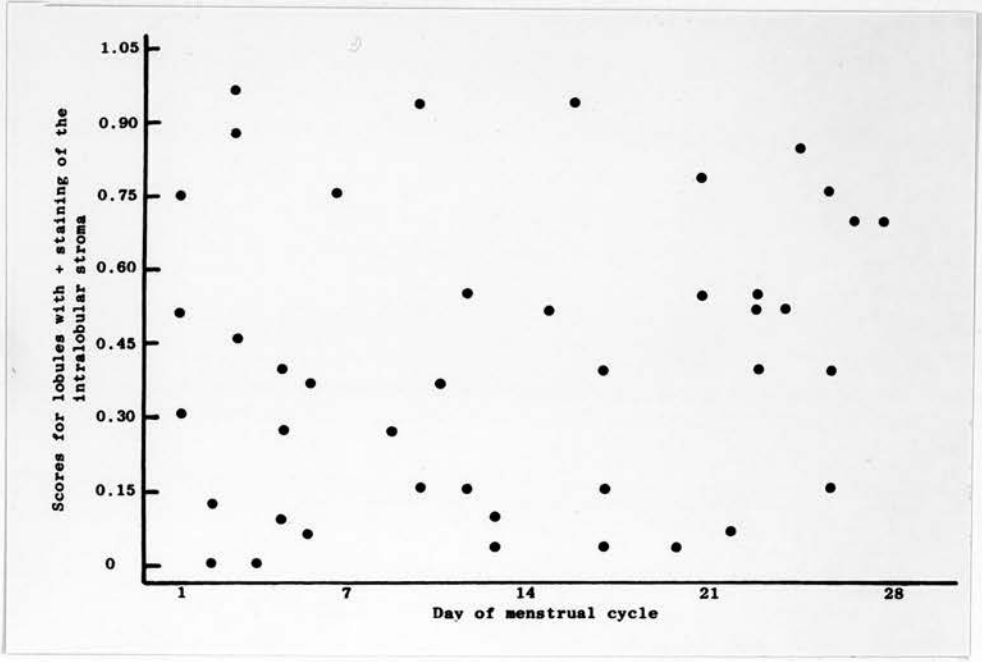
Variation in + and ++ stromal scores with day of menstrual cycle for 43 breast biopsies.

Fig. 5.7

Variation in + stromal scores with day of menstrual cycle for 43 breast biopsies.


Scores for lobules with + and ++ AB staining of the intralobular stroma
2.1
1.8
1.5
1.2
0.9
0.6
0.3
0
1
7
14
21
28
Day of menstrual cycle

5.6


Scores for lobules with + staining of the intralobular stroma
1.05
0.90
0.75
0.60
0.45
0.30
0.15
0
1
7
14
21
28
Day of menstrual cycle

5.7

+++ component was calculated as N3/NL and the results following the regression analysis are presented in Fig. 5.5. As only 9 cases had any +++ lobules and, of those, in only 5 cases did the +++ lobules exceed 5% of the total lobules scored, there were insufficient data to model any cyclical variation. All the cases with any +++ lobules were in the two weeks of the cycle following ovulation. It is suggested that they contribute to the overall cyclical variation in the combined STR score.

STR score for + and ++ lobules only

The contribution of the + and ++ components to the overall cyclical variation was assessed by calculating:-

$$\frac{(N1 \times 1) + (N2 \times 2)}{NL - N3}$$

giving a score independent of any +++ staining lobules. Following regression analysis, the results showed a cyclical variation of between $p < 0.05$ and $p < 0.10$, peaking at approximately day 26 of the cycle (Fig. 5.6).

STR score for + lobules only

The contribution of + scoring lobules to the overall cyclical variation was calculated from $\frac{N1}{N1 + NO}$ to give a score for + lobules independent of ++ and +++ lobules. Although the results following regression analysis did not show a significant cyclical variation, a trend towards higher values was observed premenstrually (Fig. 5.7).

Method for the Approximate Calculation of Day of Peak and the Confidence Interval for this Value

The STR score for all lobules (i.e. +, ++, +++, combined) showed a cyclical variation which peaked at approximately day 24 of the menstrual cycle.

The model for the cyclical regression analysis was:

$$\text{STR score} = a + b \cos\left[\frac{\text{DOC} \times 360}{28}\right] + c \sin\left[\frac{\text{DOC} \times 360}{28}\right]$$

where DOC = day of cycle.

From the regression analysis output for the STR score, the estimated day of peak EDP) and the confidence interval (CI) for this estimate were calculated as follows:

estimated c = - 0.24563

estimated b = 0.22545

standard error for c = 0.126

standard error for b = 0.120

estimated phase angle of peak = $\tan^{-1}(c/b)$

$= 47.5^0$

corresponding day of peak = $\frac{47.5 \times 28}{360} = - 3.7$

EDP = 28 - 3.7

= 24.3

EDP is approximately day 24 of the menstrual cycle. To find CI for this value, the standard deviation (s.d.) for c/b was calculated:

$$\text{s.d.} = \frac{0.126^2}{0.2254^2} + 0.120^2 \times \frac{0.126^2}{0.2254^4}$$

95% CI for c/b = (c/b) ± 2 x 0.63

= - 2.34 → 0.18

95% CI for day of peak = day 23 → day 1

This approximate method of calculation was provided by Mrs G.M. Raab, Medical Computing and Statistics Unit, University of Edinburgh.

Reproducibility of STR Score and Between Case Variation

In order to estimate the reproducibility of the STR score and the influence of quantitation error, 25 cases selected at random by an independent observer were reassessed, and the two sets of results for AB intralobular stromal staining compared (Appendix C, Table 39).

From the results, the s.d. for the two sets of STR scores were obtained:

STR score 1 = 0.618 (A)
STR score 2 = 0.621 (B)
} across cases

where STR score 1 represents the first set of measurements and

STR score 2 represents the second set of measurements

average s.d. = 0.620

and the variance $(s.d.)^2$ = 0.383

The s.d. of the STR score difference between measurements for STR score 1 and STR score 2 = 0.24.

The contribution to the variance of A and B due to quantitation error is estimated as:

$$\frac{0.24^2}{2}$$

$$= 0.028$$

(since the variance of the difference has a contribution from each of the two measurements). This is $\frac{0.028}{0.383}$ x 100 of the total variance

= 7% variance due to quantitation error.

Thus, there is no advantage in repeating the quantitation as it would only decrease the overall between specimen s.d. by a factor of $\frac{100}{107}$ (3.5% decrease).

Calculation of Within Case Variation

An estimate was obtained for the variation occurring in the results within cases as follows:

To calculate the average s.d. for within case variation:

$$\text{s.d.} = \sqrt{\frac{\Sigma_i \left[\Sigma_1^{n_j} (x_{ij} - \bar{x}_j)^2\right]}{\Sigma_j (n - 1)}}$$

where i = observations on all patients and x_{ij} = ith observations on the jth patients.

Suppose there are n_j observations on the jth patient,

$$\text{s.d.} = \sqrt{\frac{5.5842}{27}}$$

$$= 0.4548$$

$$\text{and the variance} = 0.2068$$

The between case variation = 0.383 (obtained by calculating the reproducibility of STR score for the first section of each breast). Therefore, approximately half of the between case variation is explained by within sample variability.

Effect of Age of the Patient

The influence of age was examined by dividing the results into three subgroups:

19 - 24 years (16 women)

25 - 34 years (14 women)

35 - 53 years (13 women)

Estimates of the average cyclical variations for the subgroups are given below:-

Age (in years)	No. of cases	Day of peak	STR Score	
			Peak	Trough
< 25	16	22	1.23	0.25
25 - 34	14	27	0.89	0.22
> 34	13	26	0.97	0.35

It was found that the results of the three age groups showed no significant variation between groups. However, there was some trend to more variation at a younger age, especially < 25 years.

Effect of Laterality

The effect of laterality was examined by comparing the results for biopsies from right and left breasts. No significant difference was detected and estimates for the average cyclical variation in right and left groups are given below.

	No. of cases	Day of peak	STR Score	
			Peak	Trough
Right	22	26	1.08	0.21
Left	21	22	1.19	0.52

Effect of Contraceptive Pill Usage and Parity

Analyses of the results of all lobules displaying AB staining of the intralobular stroma were undertaken to examine the effect of the use of the contraceptive pill and parity on the cyclical variation.

No significant difference was demonstrated between the results for patients using and patients not using a contraceptive pill preparation. Likewise, there was found to be no significant difference between the results for parous and nulliparous patients.

Estimations of the average cyclical variations for the various subgroups are given as follows:-

Group	No. of cases	Day of peak	STR Score	
			Peak	Trough
Pill	12	21	1.28	0.48
No Pill	31	26	0.98	0.21
Parous	20	26	0.96	0.40
Nulli-parous	23	25	1.03	0.57

It should be noted that in the comparisons of pill, parity, age and laterality, the numbers of cases in any individual group were few.

It was therefore more difficult to demonstrate significance than if larger groups had been available. Likewise, although no individual group was found to display a significant cyclical variation in itself, the numbers in any one group examined were too small for a meaningful result.

Effect of Lobule Type and Collagen Content

Photographs taken prior to processing recorded the subgross lobular morphology of the specimens. Comparisons were made between lobule type and the level of AB positivity in the intralobular stroma. It was found that no single lobule type was consistently associated with a strong AB stromal reaction. The occurrence of acid mucopolysaccharides in the intralobular stroma appeared to be independent of subgross lobule development. The use of Van Gieson's staining indicated that lobules with ++ and +++ alcianophilia of the intralobular stroma had more widely spaced collagen fibres in the intralobular stroma than other lobules, but this effect was not quantified.

Examination of Whole Breast Slices

Observation of AB/PAS sections of whole breast slices indicated that lobules showing AB positivity of the intralobular stroma occurred in a patchy nature through the tissue. Not all the lobules stained to the same intensity and +, ++ and +++ STR scores were all recorded on some slices. Unfortunately, only a limited number of slices were able to be examined in this manner as St Luke's

Hospital in Guildford was unable to provide additional technical assistance and a suitable microtome was not available in Edinburgh.

SECRETIONS

The incidence of intraluminal secretions in the ductules of lobules was examined for evidence of cyclical variation using cyclical regression analyses. As for the STR score, the secretion score (SEC score) was determined according to:-

For each section:

if NL = total number of lobules

and N1 = number of lobules containing + secretions

N2 = number of lobules containing ++ secretions

N3 = number of lobules containing +++ secretions

$$\text{then SEC score} = \frac{(N1 \times 1) + (N2 \times 2) + (N3 \times 3)}{NL}$$

Figure 5.8 plots the results following cyclical regression analysis and it was found that there was no evidence of cyclical variation in the presence of intraluminal secretions.

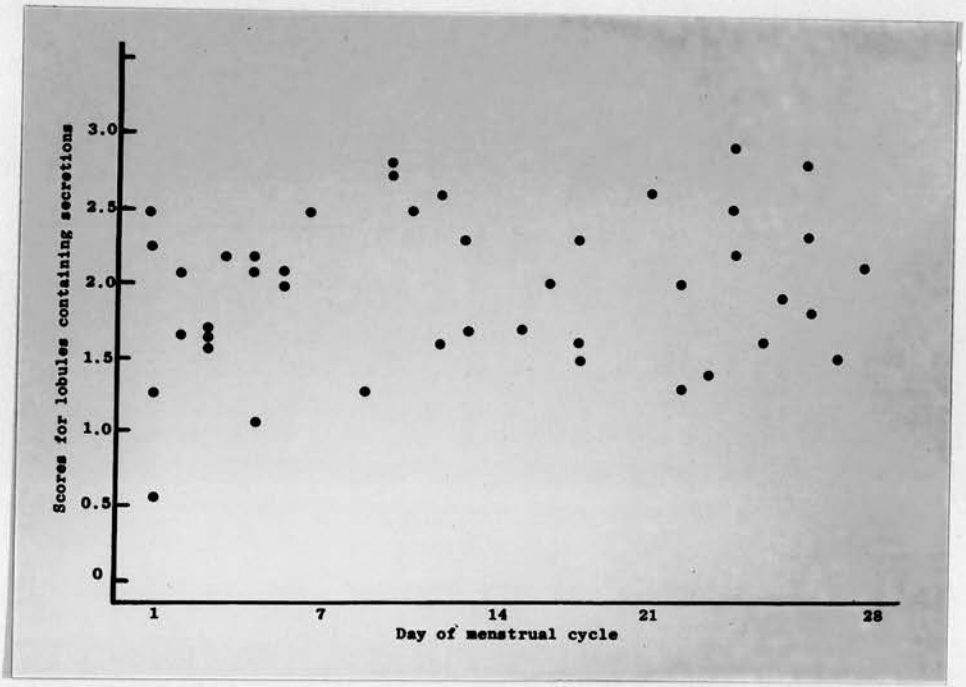
The percentage of lobules with R, R/B and N staining secretions of the total number of lobules was calculated for each case and the results for all cases analysed using cyclical regressions. Figures 5.9 - 5.11 plot the results and no cyclical variation was detected. Figure 5.12 plots the percentage of lobules not displaying secretions. Throughout the cycle, the figures are low and, excepting two cases,

Fig. 5.8

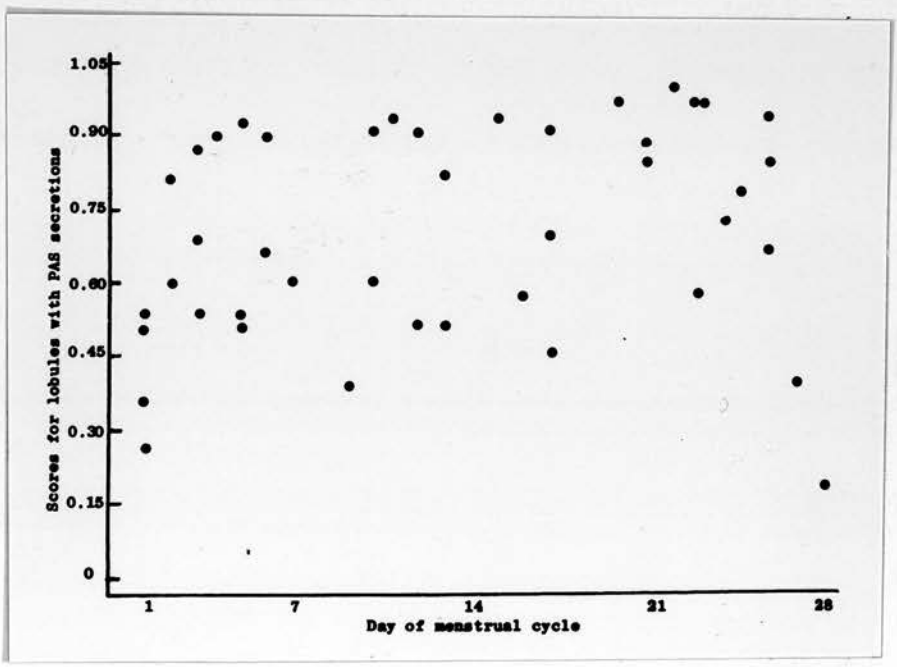
Variation in scores with day of menstrual cycle for lobules with intraluminal secretions within ductules in 43 breast biopsies.

Fig. 5.9

Variation in scores with day of menstrual cycle for lobules containing PAS secretions (R) within ductules in 43 breast biopsies.


Scores for lobules containing secretions
3.0
2.5
2.0
1.5
1.0
0.5
0
1
7
14
21
28
Day of menstrual cycle

5.8


Scores for lobules with PAS secretions
1.05
0.90
0.75
0.60
0.45
0.30
0.15
0
1
7
14
21
28
Day of menstrual cycle

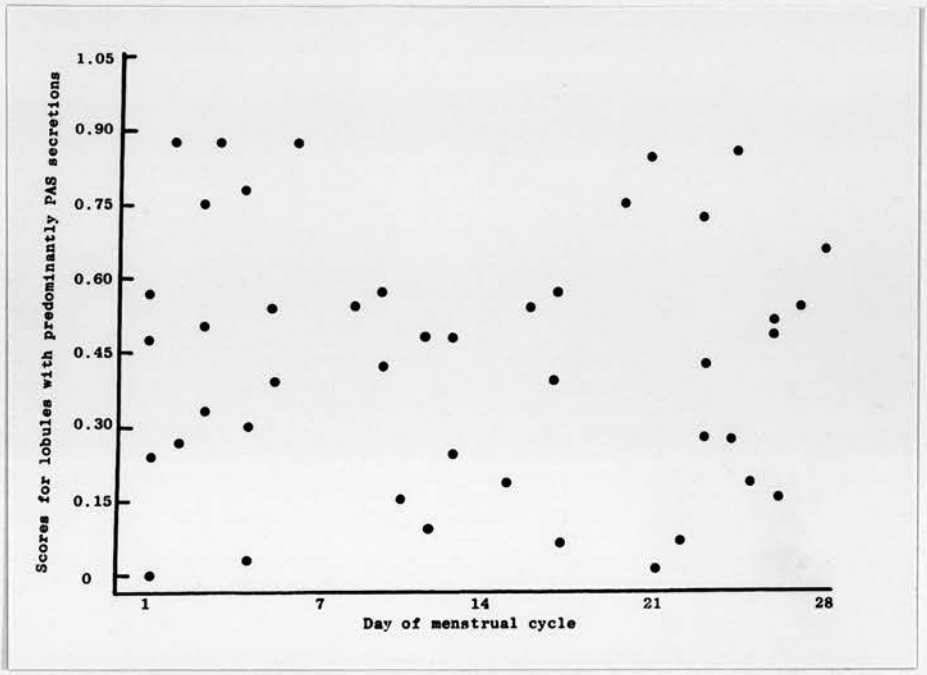
5.9

Fig. 5.10

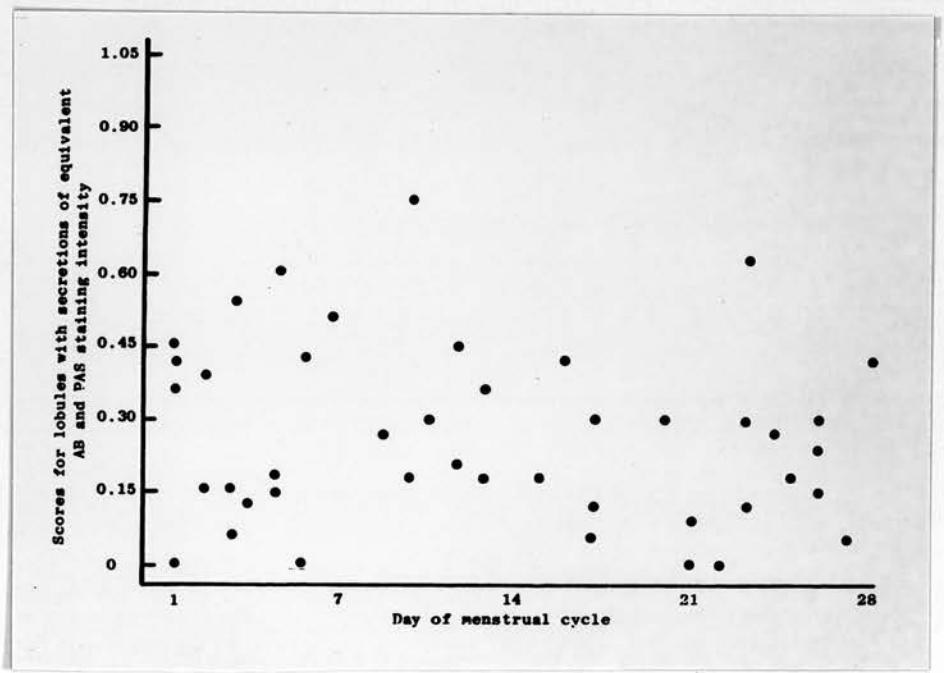
Variation in scores with day of menstrual cycle for lobules containing secretions of equivalent AB and PAS staining intensity (N) within ductules in 43 breast biopsies.

Fig. 5.11

Variation in scores with day of menstrual cycle for lobules containing predominantly PAS staining secretions (R/B) within ductules in 43 breast biopsies.


Scores for lobules with predominantly PAS secretions
1.05
0.90
0.75
0.60
0.45
0.30
0.15
0
1
7
14
21
28
Day of menstrual cycle

5·10


Scores for lobules with secretions of equivalent AB and PAS staining intensity
1.05
0.90
0.75
0.60
0.45
0.30
0.15
0
1
7
14
21
28
Day of menstrual cycle

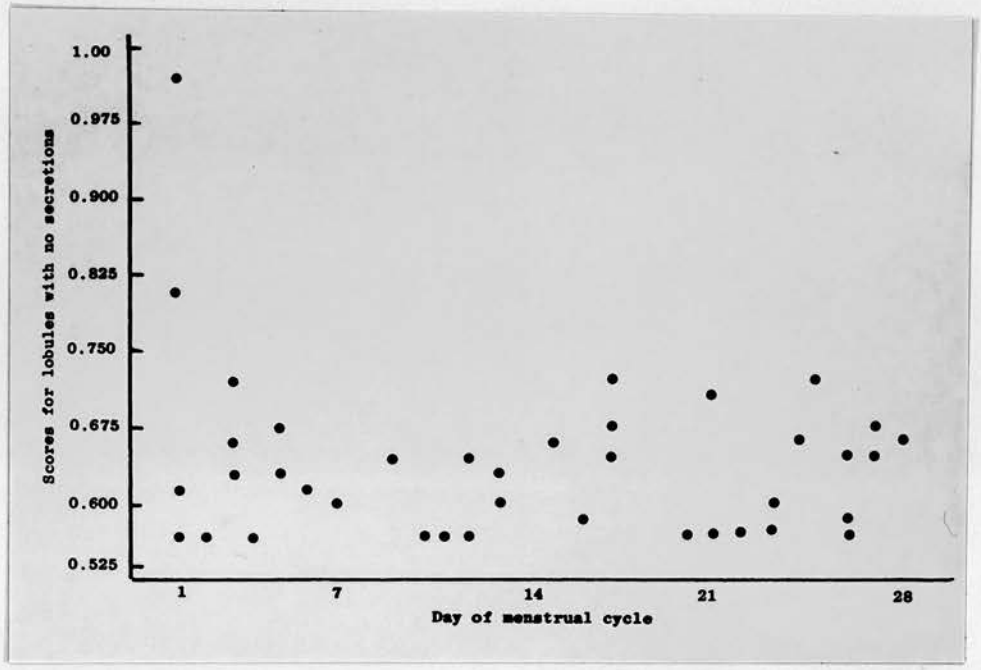
5·11

Fig. 5.12

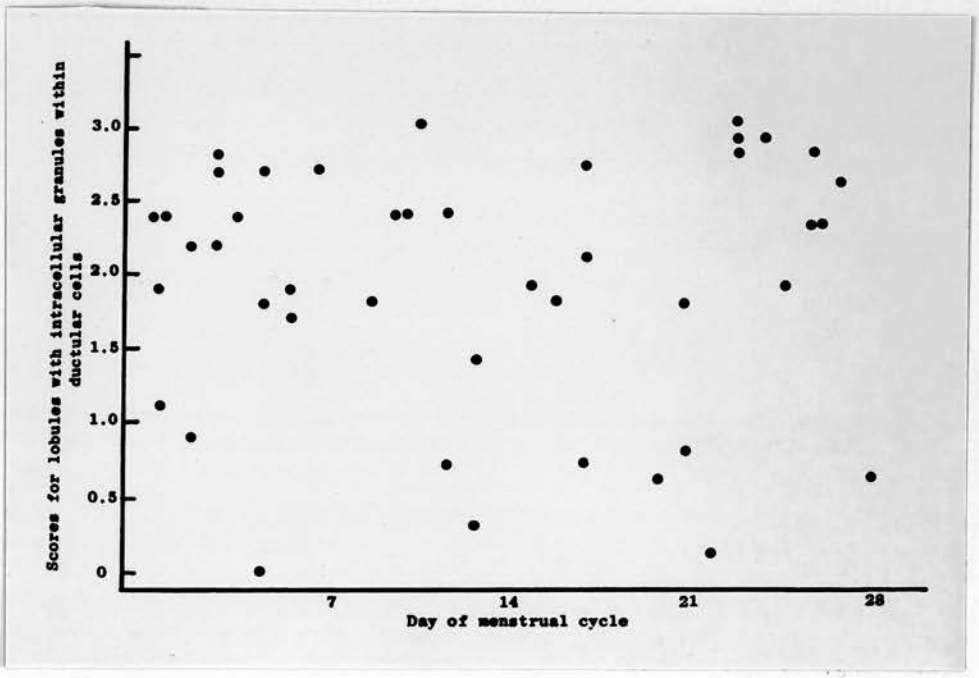
Variation in scores with day of menstrual cycle for lobules not containing intraluminal secretions within ductules in 43 breast biopsies.

Fig. 5.13

Variation in scores with day of menstrual cycle for lobules displaying intracellular granules within ductular cells in 43 breast biopsies.


Scores for lobules with no secretions
1.00
0.975
0.900
0.825
0.750
0.675
0.600
0.525
1
7
14
21
28
Day of menstrual cycle

5·12


Scores for lobules with intracellular granules within ductular cells
3.0
2.5
2.0
1.5
1.0
0.5
0
7
14
21
28
Day of menstrual cycle

5·13

are clustered about a straight line.

It was observed that both acid and neutral mucopolysaccharide staining secretions could occur within the ductules of individual lobules. The staining reactions recorded appeared to be independent of lobule type.

Diastase digestion performed on sections taken at random revealed that the intraluminal secretions were largely diastase resistant and minimal quantities of secretion were removed using this technique.

INTRACELLULAR GRANULES

The presence of granules occurring intracellularly in the luminal cells of ductules was scored as for the STR and SEC scores. Thus, the granule score (GRA score) was calculated:-

For each section:

if NL = total number of lobules

and N1 = number of lobules with + granules

N2 = number of lobules with ++ granules

N3 = number of lobules with +++ granules

$$\text{then GRA score} = \frac{(N1 \times 1) + (N2 \times 2) + (N3 \times 3)}{NL}$$

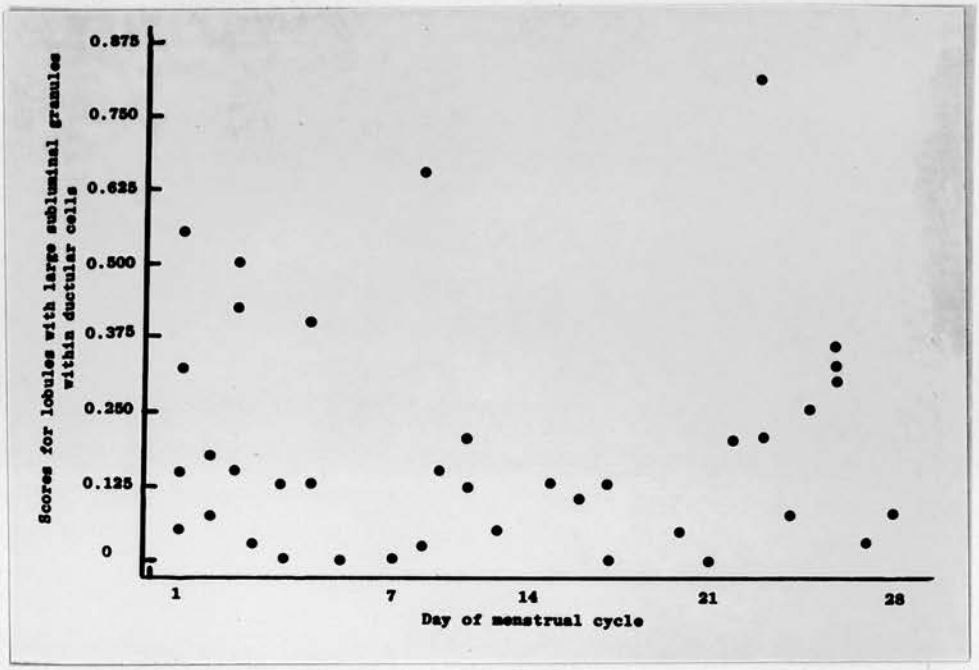
Figure 5.13 is a graph plotting the results for GRA score following cyclical regression analysis and no significant cyclical variation was detected.

Fig. 5.14

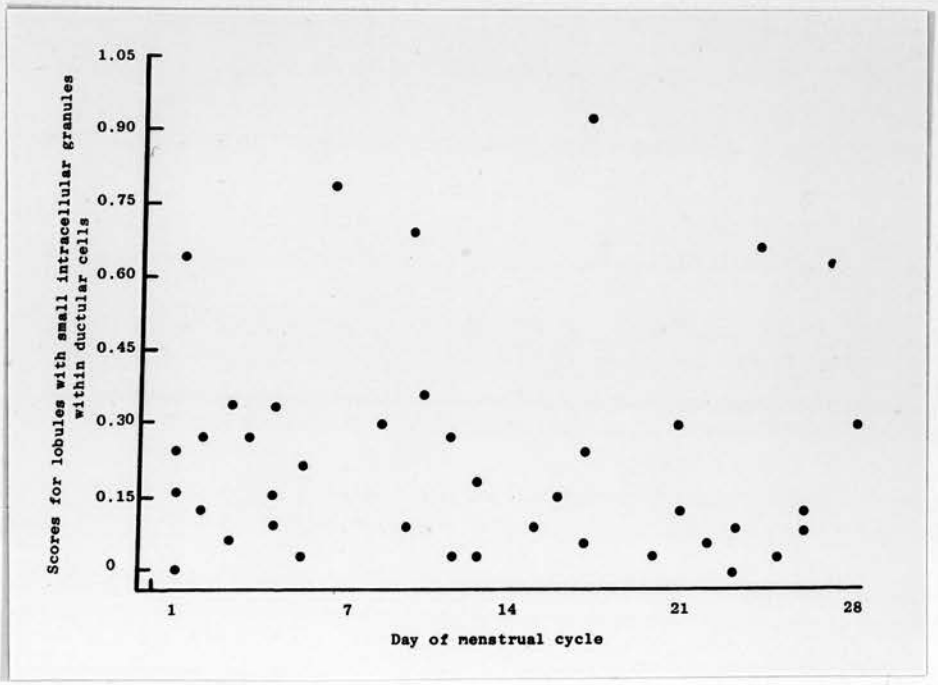
Variation in scores with day of menstrual cycle for lobules with large intracellular (subluminal) granules within ductular cells in 43 breast biopsies.

Fig. 5.15

Variation in scores with day of menstrual cycle for lobules with small intracellular granules within ductular cells in 43 breast biopsies.


Scores for lobules with large subluminal granules within ductular cells
0.875
0.750
0.625
0.500
0.375
0.250
0.125
0
1
7
14
21
28
Day of menstrual cycle

5·14


Scores for lobules with small intracellular granules within ductular cells
1.05
0.90
0.75
0.60
0.45
0.30
0.15
0
1
7
14
21
28
Day of menstrual cycle

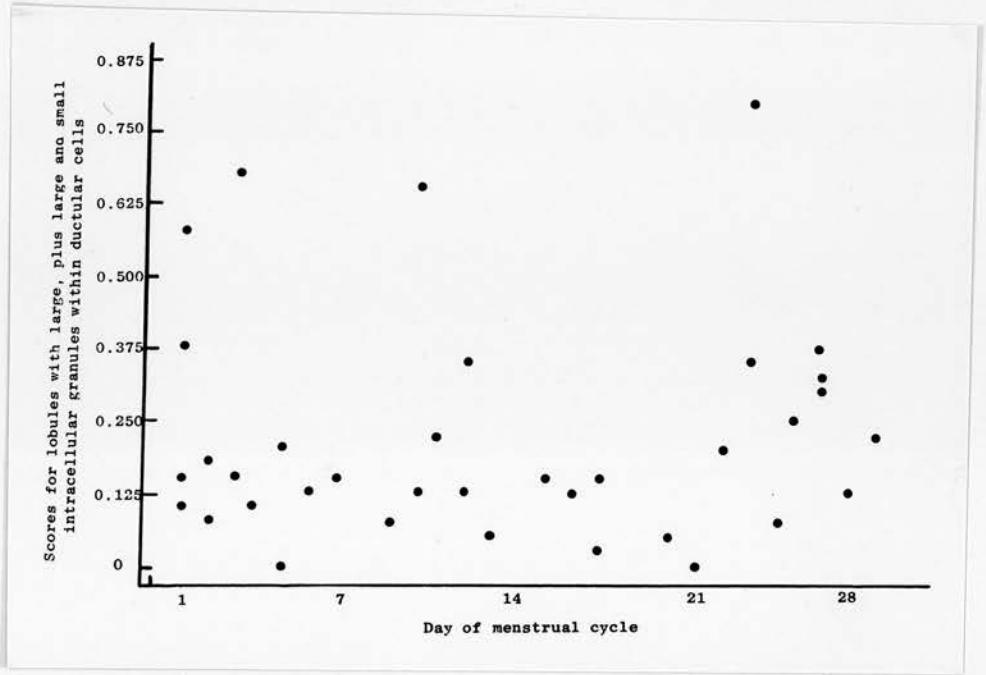
5·15

Fig. 5.16

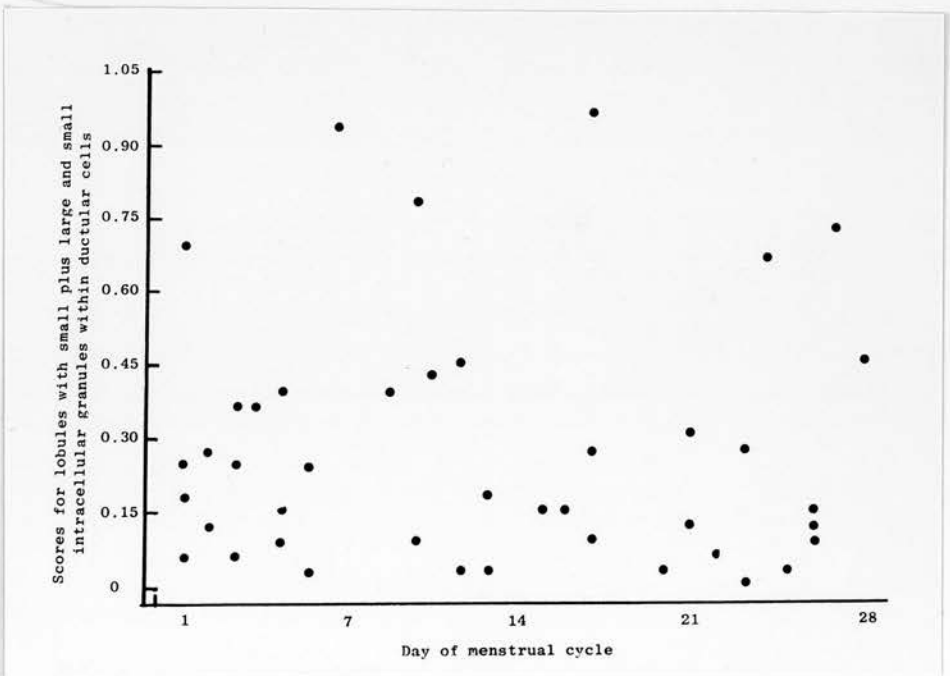
Variation in scores with day of menstrual cycle for lobules with large plus large and small intracellular granules within ductular cells in 43 breast biopsies.

Fig. 5.17

Variation in scores with day of menstrual cycle for lobules with small plus large and small cellular granules within ductular cells in 43 breast biopsies.


Scores for lobules with large, plus large and small intracellular granules within ductular cells
0.875
0.750
0.625
0.500
0.375
0.250
0.125
0
1
7
14
21
28
Day of menstrual cycle

5·16


Scores for lobules with small plus large and small intracellular granules within ductular cells
1.05
0.90
0.75
0.60
0.45
0.30
0.15
0
1
7
14
21
28
Day of menstrual cycle

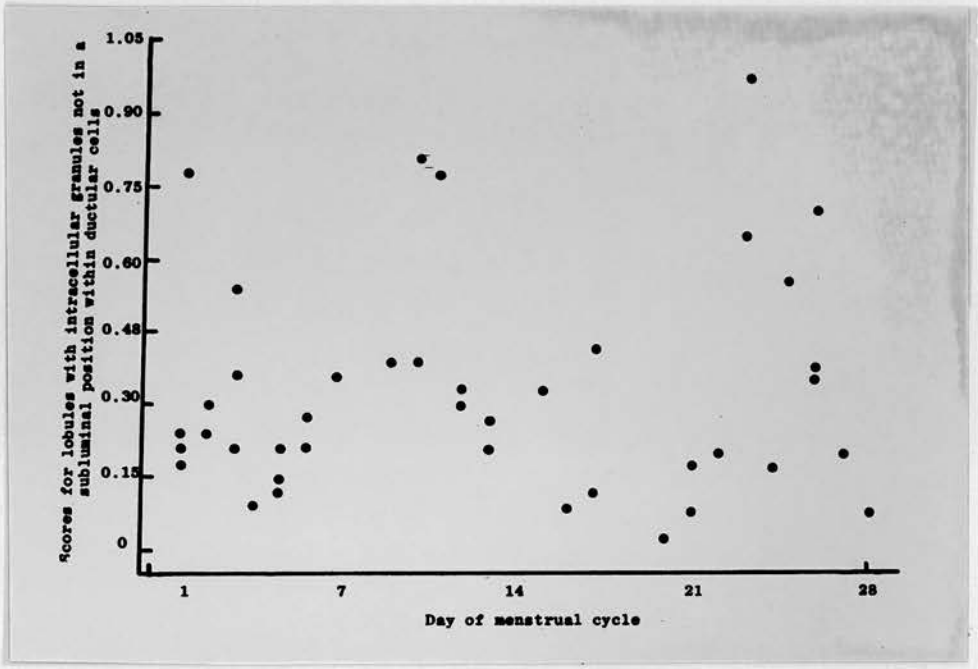
5·17

Fig. 5.18

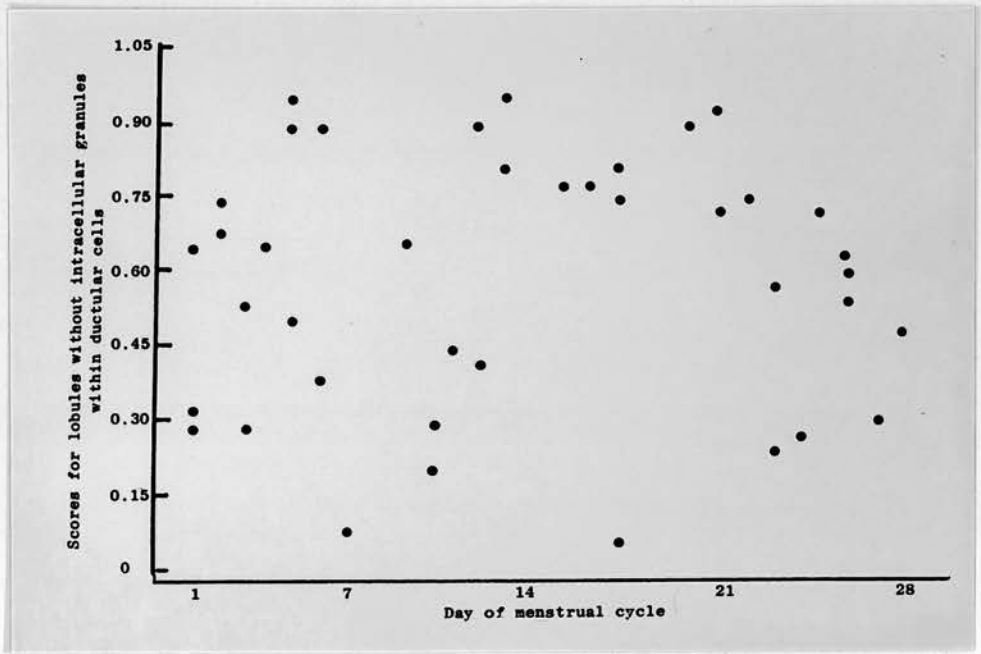
Variation in scores with day of menstrual cycle for lobules with large intracellular granules not in a subluminal position within ductular cells in 43 breast biopsies.

Fig. 5.19

Variation in scores with day of menstrual cycle for lobules without intracellular granules within ductular cells in 43 breast biopsies.


Scores for lobules with intracellular granules not in a subluminal position within ductular cells
1.05
0.90
0.75
0.60
0.45
0.30
0.15
0
1
7
14
21
28
Day of menstrual cycle

5.18


Scores for lobules without intracellular granules within ductular cells
1.05
0.90
0.75
0.60
0.45
0.30
0.15
0
1
7
14
21
28
Day of menstrual cycle

5.19

Results for the qualitative assessment of granule size and location within the cells of lobules were also analysed for any evidence of cyclical variation in the same manner as for GRA score. In a minority of lobules both large and small granules were found in a subluminal location, in which case they were assessed as a separate category. However, as this category also contributed to both large and small granules assessed individually, the following variables were examined:

i) Large subluminal granules (Fig. 5.14).

ii) Small subluminal granules (Fig. 5.15).

iii) Large plus large and small granules (Fig. 5.16).

iv) Small plus large and small granules (Fig. 5.17).

v) Large granules not in a subluminal site (Fig. 5.18).

vi) Lobules with no subluminal granules (Fig. 5.19).

In none of these analyses was there any evidence of cyclical variation. Regression analysis was not performed for large and small granules occurring together as it was clear from the results that few lobules displayed this reaction and there was no cyclical variation.

Although intracellular granules were frequently observed in ductules also displaying intraluminal secretions, the presence of granules did not always coincide with the presence of secretions. The size, quantity and location of granules appeared to be independent of subgross lobule type. The largest granules were found to range in diameter from 1 - 3.5 μm and the majority were spherical. Below 1 μm diameter, many particles could be observed but their dimensions were so small that they approached the limit of resolution of the

light microscope and accurate measurement was therefore scarcely possible.

Diastase digestion performed on sections recording high granule scores revealed that not all the granules were diastase resistant (Figs. 5.20-5.21). Granules removed by the digestion technique were not of the same size or location within cells. Neither were all the granules within any one cell always eradicated. Except very rarely, when a B/R staining reaction was recorded, all the granules stained pure magenta (R).

Fig. 5.20

Histological preparation of breast tissue (19 years). The field illustrates a duct and ductules within which PAS positive secretions and intracellular granules are clearly visible.
Stained AB/PAS. (Mag. x 250).

Fig. 5.21

Histological preparation of approximately the same field of breast tissue as that illustrated in Fig. 5.20. The tissue in this preparation has been subjected to diastase digestion followed by AB/PAS staining. The PAS positive intracellular granules are not evident. (Mag. x 250).

5·20

5·21

DISCUSSION

The results from the study of mucopolysaccharides in the breast during the menstrual cycle indicate that acid mucopolysaccharides occur in the intralobular stroma in a cyclical manner, peaking premenstrually at approximately day 24 and reaching lowest levels during mid-cycle. This result is obtained despite the fact that there is both between and within case variation. It is suggested that hyaluronic acid is the major component of the metachromatic staining reaction, although, without the use of bacterial hyaluronidase, the presence of chondroitin sulphates A and C cannot be discounted.

Current interest in the ground substance largely stems from recognition of the connective tissue as a biological system (Klemperer, Pollack & Baehr, 1941). Hyaluronic acid is a connective tissue mucopolysaccharide known to have a large water holding capacity. It is now generally believed to be synthesised by fibroblasts (Grossfield, Meyer & Goodman, 1955; Meyer *et al.*,1956; Meyer, Hoffman & Linker, 1957; Dorfman & Schiller, 1958; Whitley-Branwood, 1963; Muir, 1964; Cole & Eastoe, 1977). Mucopoly-saccharides are known to be affected by certain hormones and it is suggested that these hormones produce their effects by enhancing or depressing the activity of one or more of the enzymes involved in the synthesis or degradation of the constituent macromolecules. (Walker, 1968).

Little is established as to the influence of oestrogen on human connective tissue, but in some animals oestrogen induces an increase in the tissue content of acid mucopolysaccharides, especially hyaluronic acid, and water (McKay, 1950; Zachariae, 1958; Priest, 1961). In the sex skin of immature Macaque monkeys, oestrone produces an enormous accumulation of hyaluronate (Zuckermann, Van Wagenen & Gardiner, 1938); after oophorectomy the hyaluronate subsides and oestrogenic hormone can reactivate its production associated with a tissue oedema (Allen, 1927). The experiments of Duran-Reynals, Bunting & Van Wagenen (1950) indicate that hyaluronic acid is largely responsible for the tissue oedema and Lurie & Zappasodi (1942) reported that progesterone appeared to counteract the tissue effects of oestrone.

Further evidence of hyaluronic acid being associated with hormonal oestrogenic stimulation is found in the ovary (Jensen & Zachariae, 1958) and in the uterine cervix (Wislocki, Bunting & Dempsey, 1950). Iverson (1960) described variations in metachromasia and PAS staining occurring in the cervix during the menstrual cycle similar to those normally described for the endometrium, where there is cyclical oedema and secretion. Analysis of pre- and post-ovulatory carbohydrates in the uterine cervix show major differences between L-fucose levels, which are high in the proliferative phase, and sialic acid levels, which are high in the luteal phase of the cycle (Chantler & Debruyne, 1977).

The production of hyaluronic acid in the tissues of the ground substance may also be related to testosterone. During growth of the cock's comb, an accumulation of mucopolysaccharides, especially

hyaluronic acid, takes place in the connective tissue (Boas, 1949). Castrated male chickens (capons) fail to develop a normal comb. However, if testosterone is administered, comb growth is stimulated resulting in the appearance of hyaluronic acid in the intercellular space of the mucoid layer (Hardesty, 1931; Jacobson, 1978). The results of Dorfman & Shipley (1956) indicate that the androgen response is a local one since application of hormones directly to the comb was more effective than intramuscular injection. α-oestradiol inhibits normal but not testosterone or gonadotropin stimulated growth of the comb in chickens (Boas & Ludwig, 1950) and cortisone also inhibits the formation of mucopolysaccharides in the comb (Schiller, Benditt & Dorfman, 1952). It has been demonstrated by Doyle, Szirmai & de Tyssonsk (1964) that there is a significant correlation between hyaluronic acid content and comb weight as well as between hyaluronic acid content and water content.

Testosterone has been studied in connection with the human menstrual cycle. Lobotsky *et al.* (1964) reported that, although the range of plasma testosterone in normal women is narrow, values > 1 s.d. above the mean were observed during the ovulatory and luteal phases of the cycle. Judd & Yen (1973) found increased serum testosterone levels during the middle third of the menstrual cycle and Whitley-Branwood (1963) recorded that testosterone causes fibroblastic proliferation.

Other hormones which have been implicated in effects on acid mucopolysaccharides and hyaluronic acid in connective tissue include thyroxine (Gabrilove & Ludwig, 1957); thyrotropic hormone (Asboe-Hansen, 1960); mesenchymotropic hormone (Asboe-Hansen, 1966);

corticosteroids (Castor & Baker, 1950; Likar, Mason & Rosenkrantz, 1963; Castor, 1965) and insulin (Dorfman & Schiller, 1958). It is clear therefore that a number of hormones are capable of affecting the ground substance and causing production of acid mucopolysaccharides, in particular hyaluronic acid. In the light of the volume of evidence in the literature, some of which is conflicting, it is difficult to decide with confidence upon any individual hormone as responsible for the hyaluronic acid accumulation reported in this study.

It is inevitable that several hormones may be affecting the ground substance at any given time, since all hormones must pass through ground substance to reach the cells. Further, as each endocrine gland affects the activity of others, and a characteristic hormonal effect can only be obtained in the intact living organism, it is difficult to recognise effects using individual cells, organs or tissue slices (Asboe-Hansen, 1963).

The hormones which can affect the human menstrual cycle in the luteal phase are of most interest as a possible cause of hyaluronic acid levels peaking premenstrually. Oestradiol and progesterone both peak at approximately 5 to 8 days after mid-cycle (Ross *et al.*, 1970; Speroff & Vande Wiele, 1971). Their peak levels are just prior to that described for hyaluronic acid at day 24 in this study. It is curious that, at this point, progesterone levels are higher than those of oestradiol and that, in animals, progesterone has appeared to antagonise the tissue effects of oestrone (Gillman, 1940; Lurie & Zappasodi, 1942). However, Nizze (1972), in a study of the intralobular connective tissue in 99 healthy breasts obtained at

autopsy and 54 at biopsy, concluded that oedema of the stroma was progesterone-induced with a "permissive" effect from oestrogen, whereas oestrogen alone produced increased density of connective tissue. Bässler (1970) reported that he was unable to confirm that progesterone inhibited the mesenchymal actions of oestrogen in non-castrated rats which had not received additional progesterone during the experiment.

It may be that oestrogen as well as testosterone levels peaking late in the luteal phase (Lobotsky *et al.*, 1964) are responsible for triggering hyaluronic acid production, although Valette, Seradour & Boyer (1975) were unable to find a variation in plasma testosterone which could be related to the menstrual cycle. Prolactin may also play a contributory role. Simkin & Goodart (1960) studied prolactin activity in blood extracts. They recorded a raised prolactin activity in the second half of the cycle which was higher in nulli-parous than parous women.

There is some justification for the observation that metachromasia in the intralobular stroma varies in extent throughout the cycle. Doyle *et al.* (1964) recorded that the decline of hyaluronic acid from the rooster comb took a period of 5 to 9 days to reach half a given value. Ground substance is constantly turning over and, in the skin, the half life of hyaluronic acid is 4 days (Walker, 1968). Asboe-Hansen (1963) believed that the release of hyaluronic acid into intercellular tissues was a process which occurred continuously in the normal organism and was probably of significance in the function of the connective tissue as a water depot. Further, there are of course two peaks of oestrogen in the menstrual cycle; at approximately

day 22 and also at mid-cycle (Vande Wiele *et al.*, 1970). If it is believed that oestrogen stimulates release of hyaluronic acid into the ground substance (Ozzello & Speer, 1958), then the oestrogenic peak at ovulation should contribute to metachromasia in the intralobular stroma. A separable biphasic occurrence of metachromasia in the tissues was not demonstrated in this study and lowest levels were reached mid-cycle.

The actual mechanism of the development of tissue oedema associated with hyaluronic acid is not known. Oestrogen may be the stimulus releasing water into the tissues as it is known to affect capillary and venous permeability (Zeppa & Womack, 1962; Zeppa, 1969). However, histamine increases capillary permeability (Alksne, 1959) and is thought to cause oedema (Dale & Richards, 1918). Mast cells contain histamine and so degranulation of mast cells may be a response to histamine-liberator substances resulting in a compensating water-retention in the affected tissues (Asboe-Hansen & Wigelius, 1956). It was suggested by Alksne (1959) that at any one moment not all capillaries are equally susceptible to histamine stimulation.

Alternatively, hormonal stimulation of fibroblasts may induce the release of acid mucopolysaccharides, including hyaluronic acid, which alter the osmotic pressure of the ground substance and cause water to be drawn out of the vessels resulting in an "oedema". Fibroblasts have been described as responding to hormonal stimulation and are reported in human sexual tissues as being increased in numbers during various phases of the menstrual cycle (Dieckmann, 1925; Vogel *et al.*, 1981). The osmotic pressures of solutions containing mixtures of hyaluronic acid and albumin were measured by Laurent

& Ogston (1963). The osmotic pressures of mixed solutions were higher than the sum of the osmotic pressures of solutions of hyaluronic acid and albumin separately at the same concentrations. The effect of hyaluronic acid was reduced at low concentrations, i.e. with increased dilution. However, Gersch & Catchpole (1960) discuss why, under physiological conditions, apparently independent alterations in the water content of tissues, such as fluid fluctuations in the sex skin of the primates, can take place in tissues which are presumably in equilibrium with the plasma. They describe the ground substance as a heterogeneous colloidal system in which colloid-rich and water-rich phases can co-exist in osmotic equilibrium., the relative proportions of each helping to determine the physical consistency of a given connective tissue.

Bunting (1950) put forward the suggestion that metachromasia may not be under direct endocrine control, but rather is associated with proliferation of the stromal cells themselves. Sylvén (1945) correlated metachromasia with actively growing areas of mesenchymal tumours and Bensley (1934) described the development of metachromasia during fibrosis of the guinea pig pancreas. Measurement of mitosis and apoptosis in the ductular cells of the breast has shown that there is increased cellular activity premenstrually (Ferguson & Anderson, 1981). It is also reported by Drife (1982) that there is increased DNA synthesis in the luteal phase within ductular cells. This may be supported by increased fibroblastic activity in the stroma.

Clearly, if hormonal controls cause the development of metachromasia associated with hyaluronic acid in the intralobular

stroma, they are most probably multiple and complex. Examination of whole tissue slices suggests that the reaction is of a patchy nature throughout the breast. The effect varies between women and this is probably due to hormonal variation. It is known that there are alterations in cycle length in women and this is due to the varying number of days required for follicular growth and maturation (Speroff, Glass & Kase, 1978).

From the results, it was shown that +, ++ and +++ AB staining reactions in the intralobular stroma all contributed to the cyclical occurrence. It may be the case that, during any one cycle, some lobules are passing from no staining reaction to a + reaction; others from a + to a ++ reaction, etc., and that, for any one lobule, it may take more than 1 month to reach a +++ staining intensity which thereafter gradually subsides. Hyaluronic acid is likely to remain in the tissues for some time and alcianophilia was observed in lobules to a varying extent during all phases of the menstrual cycle.

The presence of metachromasia in the intralobular stroma could not be related to the incidence of intracellular granules or intraluminal secretions in the present study. Neither intracellular granules, nor acid and neutral mucopolysaccharide secretions showed cyclical variation. This is in contrast both to the findings of other workers (Ozzello & Speer, 1958; Bonser *et al.*, 1961) and also to the occurrence of cyclical secretions described in other sexual organs such as the endometrium.

To determine the precise nature of the intracellular granules, which were not exclusively diastase resistant as reported by Ozzello & Speer (1958), and the composition of the intraluminal

secretions, biochemical analysis is required. It is likely that both granules and secretions are composed of many substances such as glycogen, secretory IgA, lactoferrin, casein and lysosomal products (Laurence, 1978; Syré & Sehn, 1981) which are not separable with ease by histochemical techniques.

SUMMARY

Mucopolysaccharides occurring in the intralobular stroma, the lumina of ductules and the ductular cells of lobules from 43 breast biopsies have been quantified and examined for a cyclical occurrence with successive menstrual cycles. Additionally, the strength of alcian blue staining of the intralobular stroma has been related to age, laterality, contraceptive pill usage and parity.

A cyclical incidence in the strength of alcian blue staining in the intralobular stroma was recorded peaking at approximately day 24 of the cycle. There was variation both within and between cases and the patchy nature of the reaction in lobules was demonstrated by observation of large sections from whole slices of breast tissue. It is suggested that the major cause of the alcianophilia is hyaluronic acid, which is known to possess water holding capabilities, and that this may contribute to the pre-menstrual swelling and heaviness of the breasts reported by many women as a cyclical event. Alcianophilia of the intralobular stroma was not related to lobule type.

No cyclical variation was discovered in the incidence or quantity of acid and neutral mucopolysaccharide intraluminal secretions. Neither was the presence of intracellular granules within ductular cells found to be a cyclical event. The location of granules within the cells was not found to be of significance and they were not all diastase resistant. The incidence of secretions and granules was not related to subgross lobule type nor to each other.

CHAPTER 6

GENERAL CONCLUSIONS

In the present study, preparations of whole breasts and breast biopsies have been examined using a variety of techniques in an attempt to further the current understanding of the morphology of the normal human breast.

Study of the whole breast at a three-dimensional level, using subgross and duct injection techniques, established that the breast ducts do not branch in a simple dichotomous manner from the nipple to the base of the breast. Neither are the lactiferous duct systems arranged about the nipple in an ordered manner like the spokes of a wheel. Rather, they assume a layered arrangement, some main ducts extending further towards the periphery than others and having considerable overlap with neighbouring systems. Therefore, the ducts from breasts examined in the present study branched in a manner akin to that described by Cooper (1845) who stated that the ducts in the breast were mingled and interwoven with each other like a meshwork or reticulum and that individual lobes could not be distinguished or dissected out.

A curious observation, made not infrequently, was that changes occurred in the luminal diameters of ducts such that two ducts were connected by a third of smaller calibre. This was not attributable to the presence of duct ectasia in which the ducts become pathologically dilated and characteristically contain secretion. Such a condition, which is associated with benign mammary dysplasia, may partially account for this observation, particularly in the breasts of older women, but it was clearly not a contributory factor in most examples. Whether or not these alterations in duct calibre represent reaction to the varying hormonal conditions in the breast resulting

in a changed balance between parenchyma and fibrous connective tissue is speculation. Alternatively, it is not an impossibility that there are some developmental connections between lactiferous duct systems in the breast. This would appear a good histological arrangement to guarantee milk flow and allow bypass of blocked ducts during lactation. When the enormous multiplication of parenchymatous lobules occurs in pregnancy, it is unlikely, using subgross preparations of breasts (which are infrequently obtained) that the course of ducts could be established as they would be largely obscured by lobules. If there are connections between lactiferous duct systems, then such an event could afford an explanation for multifocal cancers in that cells from a primary tumour site could pass via ducts to "seed" at another site.

Unfortunately, application of duct injection techniques in the present study was limited as each breast obtained was required for several procedures, tissue from young persons was scarce, and the risks of damaging the tissue and its structures were uncertain. In future work it would be interesting to develop injection techniques further and experiment with injecting other materials such as coloured waxes, polyester resin or latex (Thompsett, 1970) and thus attempting to obtain a model to demonstrate whether connections do exist between lactiferous duct systems. It may also be profitable to apply mathematical methods to study the branching of ducts in order to establish whether the breast has an ordered branching arrangement or whether the branching patterns are predominantly random. The branching angles of ducts could be measured to determine whether they relate to the theories of flow which have

been described for other branching systems such as arteries and trees (Murray, 1926, 1927). Ease of flow within the duct system in the breast becomes an important factor when the organ is fully functional during lactation.

Quantitation of the breast revealed that the upper outer quadrant does not consistently contain the greatest number of parenchymatous lobules. This is not the finding that was anticipated by other workers (e.g. Haagensen, 1971; Vorherr, 1974). The quantity of lobules could not be predicted from the amount of the non-fatty component, which is in essence the fibrous stroma. The majority of lobules were found to lie within the fibrous connective tissue and frequently lobules occurred in focal areas of high density. Most were found at approximately the junction of the middle and deep thirds in the breast and not therefore in the most basal portion.

Supporting Cooper (1845), lobular distribution within the fibrous stroma could be described as similar to the petals of a flower, the spaces between petals being occupied by fat. However, just as variation between species of flowers is enormous, variation between breasts is great and rules governing the distribution of lobules could not be made. Generalisations were possible and an 'ideal' statistical model was constructed which indicated an eccentric distribution of lobules favouring the upper half of the breast, whereas the non-fatty component favoured an elliptical distribution with the greatest portion of tissue occurring in the upper outer quadrant. Although lobule numbers decreased significantly with age, there was no statistical relationship between the

number of lobules present and either parity, laterality or tumour quadrant. The results suggested that the incidence of tumours is not related to the quadrant having the greatest quantity of either lobules or fibrous stroma. It seems most likely that tumours arise randomly and are more common in the upper outer quadrant because this quadrant is generally the largest and, over a large series of specimens, will therefore probably record a higher incidence of tumour occurrence.

In order to advance understanding of the lobular distribution beyond the present study, a great many more specimens would require to be examined, preferably by a modified and shortened technique. Arguably, the most significant outcome of this aspect of the research is the conclusion that biopsies are unlikely to provide sufficient information to predict lobule numbers, a finding in agreement with the like observation of Parks (1959). The present study highlights the complexity of a quantitative approach to the breast as not only the number but also the size of lobules varies and, additionally, the whole organ must be assessed.

The study of mucopolysaccharides in the breast confirmed the subjective finding of Ozzello & Speer (1958) that acid mucopolysaccharides occur in the intralobular stroma in a cyclical manner, in the present study peaking premenstrually at approximately day 24. Observation of alcian blue positivity of the intralobular stroma on large tissue sections indicated a focal distribution present to some extent in the majority of sections studied. It is suggested that the reaction may not develop and disappear every cycle, but take several cycles to reach its full potential and thereafter several

cycles to regress. The patchy nature of the reaction accounted for the variation observed between different blocks of tissue from the same biopsy. However, rather than confirm the validity of the finding on a second series of breast biopsies, it would be more rewarding to repeat the experiment using large tissue sections. If breasts were selected representing various phases of the menstrual cycle and the majority of the constituent slices were examined, then a very good assessment of acid mucopolysaccharide activity in the breast and its variation with the menstrual cycle would be obtained. An investigation on this scale was unfortunately beyond the scope of this study as the necessary equipment was unavailable. Alternatively, applying biochemical techniques to fresh tissue may be sufficient to confirm the presence of hyaluronic acid and its cyclical fluctuation in the intralobular breast stroma; but if biopsies were used this would not be representative of the activity in the whole breast.

It is apparent from the present study that the presence of secretions in ductules is uniform throughout the menstrual cycle and is without cyclical variation. Intracellular granules within ductular cells were also found to have no cyclical incidence. However, a qualitative analysis of the composition of both secretions and granules was not attempted and is a subject for future experiment, especially as Petrakis (1977) suggests that oestrogen and other hormones may act to increase cancer risk indirectly through stimulatory activity on ductal and lobular development, and epithelial secretory activity. Further, he states that the rate of breast fluid secretion and reabsorption could determine the extent

and exposure of the breast epithelium to secreted environmental and endogenous carcinogens. Thus, secretory activity of the non-lactating breast could provide mechanisms for initiating and promoting factors acting on the epithelium to produce atypical, premalignant and possibly malignant cells.

Normal subgross lobule types in the breast have not been classified with sufficient detail by workers in the past. The results in this study indicate that their occurrence varies with age. The incidence of solid lobules decreased whereas that of other lobule types increased with age. The histological characteristics of the lobule types remains to be detailed and is a project of some importance if the boundaries of normality are to be properly defined and if the physiological activity of the normal breast is to be understood more completely. If there is a spectrum of lobule types present at any one time in the breast, then it should be appreciated with clarity which lobules are developing and which are regressing. It is a possibility that lobules develop and regress not during a single menstrual cycle but over a series of cycles. In addition, the potential of the breast to develop mature lobules may decrease with age, thus explaining the higher incidence of other than solid lobule types with increasing age. It was interesting that parity did not affect the results in the present study. A range of lobule types was seen in nulliparous as well as parous breasts suggesting a return to 'normality' following childbirth and lactation. However, the influence of age on the series of breasts examined may have prejudiced this observation. Clearly, more research is required into normal lobule types in the breast, more especially as the

pathological subgross lobule types have been comparatively well defined.

It may be rewarding to conduct a more extensive comparison than that made in this study between subgross lobule types and the incidence of alcian blue positivity in the intralobular stroma, intraluminal secretions and intracellular granules in ductules, in an attempt to correlate lobular activity with lobule type. Apoptosis and mitotic activity as markers of cyclical variation are described in the work of Ferguson & Anderson (1981) and may also be compared to lobule type over a series of specimens.

Comparison of breast slices and matching radiographs revealed that the parenchymal content of the breast could not be predicted from radiographic appearances alone. Neither could other factors be predicted with confidence from the radiographs such as subgross lobule type, parity or age and the fibrous stroma largely dictated radiographic opacities and patterns. The thinner the slice of breast tissue, the more readily were parenchymatous lobules distinguished, having mottled or 'fluffy' opacities. It is suggested that, as there is such difficulty in determining the parenchymatous structures from 2 mm thick slices of tissue, then the ease with which they may be recognised in mammograms as indicated by Wolfe (1976a,b) amongst many other researchers is debatable. In future work, comparisons between subgross radiographic and mammographic appearances may be valuable to refute or confirm the validity of mammographic classifications such as those of Wolfe (1976a,b).

As Short & Drife (1977) suggest, in order to understand the aetiology of mammary cancer, it is first necessary to have a clear understanding of the physiology of normal mammary development and lactation. They stress that the epidemiological evidence points to a major role of steroid hormones in the development of mammary carcinoma, citing the low incidence in males and the protective effect of ovariectomy in females as support for this statement.

The importance of defining normal is further highlighted in a review article compiled by Ernster *et al.* (1981) who state that different investigations have concluded either that benign breast disease displays impressive epidemiological similarity to breast carcinoma or that there are not sufficient epidemiological differences to assume the two conditions are aetiologically distinct. The lack of clarification of benign breast disease is alluded to by Golinger (1978) who states that as yet little is known about the pathophysiology of benign disease of the breast and even less pertinent information has been scientifically acquired; the prevalence of fibrocystic mastopathy is unknown but it occurs at all ages after menarche. Haagensen (1971) and Rush (1974) emphasise that physiological changes taking place in the breast are often mistaken for disease of the breast. Urban (1967), commenting on cancer of the breast, states that:

> Since the stage of the disease at the time of definitive treatment is the most important factor in prognosis, it is absolutely necessary to make all efforts to detect cancer of the breast in its early clinical stages.

The present study supports the conclusions of Parks (1959) who maintains that, by using thick sections of tissue,

> a far greater quantity of tissue can be measured than is practicable with paraffin sections and furthermore it can be examined serially. A more comprehensive impression is therefore attained than is possible from the study of thin sections. This is particularly true of an organ such as the breast in which there is great variation from patient to patient and even from one segment to another in the same person. A biopsy from one part of the breast therefore gives no reliable information about the state of the remainder. Biopsies removed at different periods within the menstrual cycle are likewise of little value in obtaining information about cyclical breast changes. For similar reasons it is difficult to tell what is normal and what is abnormal. It is impossible for instance to estimate the normal density of lobules.

It is suggested that subgross techniques can be used profitably to extend the knowledge of normal human breast morphology which, sadly, is still far from comprehensive and a long way from complete.

It is worth reflecting on the comment of Sir Hedley Atkins (1967) who, in a lecture on the work of Sir Astley Cooper, said that over the succeeding years, Cooper's description of the breast has only needed trivial modification. If this is so, then Cooper's work is not adequately recognised, and the comment stands perhaps as an indictment of those of us who have followed for, with all the techniques at our disposal, we apparently have so few advances to offer and such poor unity of opinion over our data.

BIBLIOGRAPHY

AHERNE, W. (1970). Quantitative methods in histology. *Journal of Medical Laboratory Technology*, 27, 160-170.

AHRÉN, K. & JACOBSOHN, D. (1956). Mammary gland growth in hypophysectomized rats injected with ovarian hormones and insulin. *Acta Physiologica Scandinavica*, 37, 190-203.

ALKSNE, J.F. (1959). The passage of colloidal particles across the dermal capillary walls under the influence of histamine. *Quarterly Journal of Experimental Physiology*, 44, 51-66.

ALLEN, E. (1927). The menstrual cycle of the monkey, *macacus rhesus;* observations of normal animals, the effects of removal of the ovaries and the effects of injections of ovarian and placental extracts into spayed animals. *Contributions to Embryology*, 19, 1-44

ALLISON, R.T. (1973). The effects of fixation on the subsequent demonstration of mucopolysaccharides. *Medical Laboratory Technology*, 30, 27-31.

ALVIM, P. de T. (1964). Tree growth periodicity in tropical climates. In: *The Formation of Wood in Forest Trees, edited by Zimmerman, M.H.* pp. 479-495. Academic Press, New York.

ANDERSSON, I., ANDRÉN, L. & PETTERSSON, H. (1978) Influence of age at first pregnancy on breast parenchymal pattern: a preliminary report. *Radiology*, 126, 675-676.

ASBOE-HANSEN, G. (1960). The pathogenesis of cutaneous myxedema. *Archives of Dermatology*, 82, 32-34.

ASBOE-HANSEN, G. (1963). The hormonal control of connective tissue. *International Review of Connective Tissue Research*, 1, 29-61.

ASBOE-HANSEN, G. (1966). Hormone control of connective tissue. *Federation Proceedings,* 25, 1136-1139.

ASBOE-HANSEN, G. & WIGELIUS, O. (1956). Histamine and mast cells. Studies on living connective tissue in the hamster cheek pouch. *Acta Physiologica Scandinavica,* 37, 350-358.

ATKINS, Sir H. (1968). Astley Cooper and diseases of the breast. *Guy's Hospital Reports,* 117, 199-206.

AZZOPARDI, J.G. (1979). Problems in Breast Pathology. *Major Problems in Pathology, Vol. II.* W.B. Saunders Co. Ltd., London.

BAILEY, H. (1977). Bailey and Love's Short Practice of Surgery. 17th Ed. p. 633. H.K. Lewis & Co. Ltd., London.

BAIN, C., WILLETT, W., ROSNER, B., SPEIZER, F.E., BELANGER, C.& HENNEKENS, C.H. (1981). Early age at first birth and decreased risk of breast cancer. *American Journal of Epidemiology,* 114, 705-709.

BÄSSLER, R. (1970). The morphology of hormone induced structural changes in the female breast. *Current Topics in Pathology,* 53, 1-89.

BENSLEY, S.H. (1934). On the presence, properties and distribution of the intercellular ground substance of loose connective tissue. *Anatomical Record,* 60, 93-111.

BERBERICH, J. & JAFFÉ, R. (1924). Der lipoidstoffwechsel der ovarian mit besonderer berücksichtigung des menstruationszyklus nebst untersuchungen am nebennieren und mamma. *Zeitschrift für die gesamte Anatomie,* 10, 1-27.

BERKA, F. (1911). Die brustdrüse verschiedener altersstufen und während der schwangerschaft. *Frankfurter Zeitschrift für Pathologie,* 8, 203-256.

BERNFIELD, P. (1951). Enzymes of starch degredation and synthesis. *Advances in Enzymology,* 12, 379-428.

BOAS, N.F. (1949). Isolation of hyaluronic acid from the cock's comb. *Journal of Biological Chemistry,* 181, 573-575

BOAS, N.F. & LUDWIG, A.W. (1950). The mechanism of estrogen inhibition of comb growth in the cockerel with histological observations. *Endocrinology,*46, 299-306.

BOATMAN, E.S. & LOWE, D. (1971). Photographic mapping of a tissue surface to locate fields for electron microscopy; mouse lung. *Stain Technology,*46, 63-69.

BONSER, G.M. (1936). The effect of oestrone administration on the mammary glands of male mice of two strains differing greatly in their susceptibility to spontaneous mammary cancer. *Journal of Pathology and Bacteriology,* 42, 169-181.

BONSER, G.M., DOSSETT, J.A. & JULL, J.W. (1961). Human and Experimental Breast Cancer. Pitman, London.

BOYD, N.F., O'SULLIVAN, B., CAMPBELL, J.E., FISHELL, E., SIMOR, I., COOKE, G. & GERMANSON, T. (1982). Mammographic signs as risk factors for breast cancer. *British Journal of Cancer,* 45, 185-193.

BREBNER, D.M., EPSTEIN, E.E. & LANGE, M. (1978). Xerographic parenchymal patterns and breast cancer. *South African Medical Journal,* 54, 853-856.

BREM, S.S., JENSEN, H.M. & GULLINO, P.M. (1978). Angiogenesis as a marker of preneoplastic lesions of the human breast. *Cancer,* 41, 239-244.

BRENNAN, M.J. (1980). Etiology and pathophysiology of breast cancer. In: *Breast Cancer: New Concepts in Etiology and Control, edited by Brennan, M.J., McGrath, C.M., Rich, M.A.* pp. 3-16. Academic Press Inc., New York, London.

BRISSON, J., MERLETTI, F., SADOWSKY, N.L., TWADDLE, J.A., MORRISON, A.S. & COLE, P. (1982a). Mammographic features of the breast and breast cancer risk. *American Journal of Epidemiology*, 115, 428-437.

BRISSON, J., SADOWSKY, N.L., TWADDLE, J.A., MORRISON, A.S., COLE, P. & MERLETTI, F. (1982b). The relation of mammographic features of the breast to breast cancer risk factors. *American Journal of Epidemiology*, 115, 438-443.

BRUCE, J. & RUSSELL, F.M. (1962). Premenstrual tension: study of weight changes and balances of water, sodium and potassium. *Lancet*, 2, 267-271.

BRUCE, Sir J., WALMSLEY, R.& ROSS, J.A. (1964). Manual of Surgical Anatomy. p. 177. E. & S. Livingstone Ltd., Edinburgh, London, New York.

BUNTING, H. (1950). The distribution of acid mucopolysaccharides in mammalian tissues as revealed by histochemical methods. *Annals of The New York Academy of Sciences*, 52, 977-982.

BUSK, T. & CLEMMESEN, J. (1947). The frequencies of left and right sided breast cancer. *British Journal of Cancer*, 1, 345-351.

BYRD, B.F. (1978). Incidence and mortality of breast cancer. In: *The Breast, edited by Gallager, H.S., Leis, H.P. Jr., Snyderman, R.K., Urban, J.A.* pp. 33-36. The C.V. Mosby Company, Saint Louis.

CAMERON, A.M. & FAULKIN, L.J. (1974). Subgross evaluation of the non-human primate mammary glands: method and initial observations. *Journal of Medical Primatology*, 3, 298-310.

CAMPBELL, H. & TOMKEIEFF, S.I. (1952). Calculation of the internal surface of a lung. *Nature*, 170, 117.

CARSON, F.L., MARTIN, J.H.& LYNN, J.A. (1973). Formalin fixation for electron microscopy. A re-evaluation. *American Journal of Clinical Pathology,* 59, 365-373.

CASTOR, C.W. (1965). The effects of chronic glucocorticoid excess on human connective tissue cells *in vitro*. *Journal of Laboratory and Clinical Medicine,* 65, 490-499.

CASTOR, C.W. & BAKER, B.L. (1950). The local action of adrenocortical steroids on epidermis and connective tissue of the skin. *Endocrinology,* 47, 234-241.

CERIANI, R. (1974). Hormones and other factors controlling growth in the mammary gland: a review. *Journal of Investigative Dermatology,* 63, 93-108.

CHAFFE, A., ROEBUCK, E.J. & WORTHINGTON, B.S. (1979). Observer assessment of mammograms and an evaluation of the significance of radiographic patterns. *British Journal of Radiology,* 52, 347.

CHALKLEY, H.W. (1943). Method for the quantitative morphologic analysis of tissues. *Journal of the National Cancer Institute,* 4, 47-53.

CHANTLER, E. & DEBRUYNE, E. (1977). Factors regulating the changes in cervical mucus in different hormonal states. In: *Mucins in Health and Disease. Advances in Experimental Medicine and Biology, Vol. 89, edited by Elstein, M, & Parke, D.V.* pp.131-141. Plenum Press, New York, London.

CHATTERTON, R.T. Jr. (1978). Mammary gland: development and secretion. *Obstetrics and Gynecology Annual,* 7, 303-324.

CHEATLE, G.L. & CUTLER, M. (1931). Tumours of the Breast. Arnold, London.

CHECK, W.A. (1980). Can mammographic parenchymal patterns foretell breast cancer? *Journal of the American Medical Association*, 244, 221-224.

CHESTERMAN, W. & LEACH, E.H. (1949). Low viscosity nitrocellulose for embedding tissues. *Quarterly Journal of Microscopical Science*, 90, 431-434.

CHOI, N.W., HOWE, G.R., MILLER, A.B., MATTHEWS, V., MORGAN, R.W., MUNAN,L., BURCH J.D., FEATHER, J., JAIN, M.& KELLY, A. (1978). An epidemiologic study of breast cancer, *American Journal of Epidemiology*, 107, 510-521.

CLEMMESEN, J. (1948). Carcinoma of the breast. Symposium. 1. Results from statistical research. *British Journal of Radiology*, 21, 583-590.

COLE, A.S. & EASTOE, J.E. (1977). Biochemistry and Oral Biology. p. 310. John Wright and Sons Ltd., Bristol.

COOK, H.C. (1972). Human Tissue Mucins. Butterworth & Co. Ltd., London.

COOK, H.C. (1982). Carbohydrates. In: *History and Practice of Histological Techniques, 2nd Ed. Bancroft, J.D. & Stevens, A.* pp. 180-216. Churchill Livingstone, Edinburgh.

COOPER, Sir A.P. (1845). The Anatomy and Diseases of the Breast. Lea and Blanchard, Philadelphia.

COOPER, D.J. (1974). Mucin histochemistry of mucous carcinomas of breast and colon and non-neoplastic breast epithelium. *Journal of Clinical Pathology*, 27, 311-314.

CORREA, P. (1975). Epidemiology of cancer of the breast. *American Journal of Clinical Pathology*, 64, 720-727.

COWIE, A.T. (1947). The role of the adrenal cortex in mammary development and milk secretion. Ph.D. Thesis, University of Reading.

CULLING, C.F.A. (1963). Handbook of Histopathological Techniques, 2nd Ed. p. 201. Butterworths, London.

CUMMINS, H. (1943). The skin and mammary glands. In: *Morris' Human Anatomy. A Complete Systematic Treatise. 10th Ed , edited by Schaeffer, J.P.* pp. 53-76. The Blakiston Company, Philadelphia.

CUTLER, M. (1962). Tumours of the Breast. Their Pathology, Symptoms, Diagnosis and Treatment. Pitman Medical Publishing Co. Ltd., London.

DABELOW, A. (1957). Die milchdrüse. In: *Handbuch der Mikroskopischen Anatomie des Menschen. Vol. 3, pt. 3, haut und sinnesorgane, edited by Von Moellendorff, W. and Bargmann, W.* pp. 277-485. Springer-Verlag, Berlin, Gottingen, Heidelberg.

DALE, H.H.& RICHARDS, A.N. (1918). Pharmacology of histamine. *Journal of Physiology,* 52, 110-165.

DAVIES, J.D., ROBERTS, G. & RICHARDSON, P.J. (1973). A serial whole-organ slicing technique for examining surgically resected breasts. *Journal of Clinical Pathology,* 26, 891-892.

DAWSON, E.K., (1934). A histological study of the normal mamma in relation to tumour growth. 1. Early development to maturity. *Edinburgh Medical Journal,* 41, 653-682.

DAWSON, E.K. (1935a). A histological study of the normal mamma in relation to tumour growth. II. The mature gland in pregnancy and lactation. *Edinburgh Medical Journal,* 42, 569-598.

DAWSON, E.K. (1942). Mammary cancer and the menopause. *Edinburgh Medical Journal*, 50, 721-736.

DEATON, W.R. Jr., BRADSHAW, H.H. & WINSTON-SALEM, N.C., (1950). Carcinoma of the breast - why the upper outer quadrant? *Surgery*, 28, 583-584.

DEMING, W.E. (1964). Statistical Adjustment of Data. p. 30. Dover Publications Inc. New York.

DICKSON, L.M. & HEWER, E.E. (1950). The structure of the breast. In: *The Breast: Structure, Function, Disease, edited by Saner, F.D.* pp. 1-52. John Wright and Sons Ltd., Bristol.

DIECKMANN, H. (1925). Ueber die histologie der brustdrüse bei gestörtem und ungestörtem menstruationsablauf. *Virchows Archiv für pathologische Anatomie und Physiologie und für klinische Medizin*, 256, 321-356.

DIXON, R. (1981). The mathematical daisy. *New Scientist*, 92, 792-795.

DIXON, W.J., BROWN, M.B., ENGELMAN, L., FRANE, J.W., HILL, M.A., JENNRICH, R.I. & TOPOREK,J.D. (1981). BMDP Biomedical Computer Programs P-series. pp. 237-250. University of California Press. Berkely, Los Angeles, London.

DOBRETSBERGER, W. (1967). Die nicht pathologische brustdrüse und ihre mannigfaltigkeit. *Journal de Radiologie et d'Electrologie et de Medecine Nucleaire*, 48, 748-750.

DONEGAN, W.L. (1967). Diagnosis of mammary cancer. In: *Cancer of the Breast by Spratt, J.S. Jr., Donegan, W.L.* pp. 34-69. W.B. Saunders Co. Ltd., London.

DORFMAN, A. & SCHILLER, S. (1958). Effects of hormones on the metabolism of acid mucopolysaccharides of connective tissue. *Recent Progress in Hormone Research,* 14, 427-456.

DORFMAN, R.I. & SHIPLEY, R.A. (1956). Androgens. Biochemistry, Physiology and Clinical Significance. p. 545. John Wiley & Sons Inc., New York.

DOYLE, J., SZIRMAI, J.A. & de TYSSONSK, E.R. (1964). Connective tissue changes in the rooster comb during regression. *Acta Endocrinologica,* 45, 457-475.

DRIFE, J.O. (1982). The effects of parity and the menstrual cycle on the normal mammary gland and their possible relationship to malignant change. M.D. Thesis, University of Edinburgh.

DRURY, R.A.B. & WALLINGTON, E.A. (1967). Carleton's Histological Technique. 4th Ed. p.129. Oxford University Press, New York, Toronto.

DUNNILL, M.S. (1968). Quantitative methods in histology. In: *Recent Advances in Clinical Pathology, edited by Dyke, S.C.* pp. 401-416. J. & A. Churchill Ltd., London.

DURAN-REYNALS, F., BUNTING, H. & VAN WAGENEN, G. (1950). Studies on the sex skin of *macaca mulatta*. *Annals of The New York Academy of Sciences,* 52, 1006-1014.

EASTGATE, R.J., GILCHRIST, K.W. & MATALLANA, R.H. (1979). Enhancement of tissue structure visualisation in breast specimen radiography. *Radiology,* 132, 744-746.

EGAN, R.L. (1964). Present status of mammography. *Annals of The New York Academy of Sciences,* 114, 794-802.

EGAN, R.L. (1970). Mammography and Breast Diseases. *Golden's Diagnostic Radiology, Section 19.* Williams & Wilkins, Baltimore.

EGAN, R.L. (1972). Mammography. 2nd Ed. Charles C.Thomas, Springfield, Illinois.

EGAN, R.L., ELLIS, J.T. & WALDO POWELL, R. (1969). Team approach to the study of diseases of the breast. *Cancer,* 23, 847-854.

EGAN, R.L. & MOSTELLER, C. (1977). Breast cancer mammography patterns. *Cancer,* 40, 2087-2090.

EGAN, R.L. & McSWEENEY, M.B. (1979). Mammographic parenchymal patterns and risk of breast cancer. *Radiology,* 133, 65-70.

ENGEL, S. (1941). Anatomy of lactating breast. *British Journal of Children's Diseases,* 38, 14-21.

ENGLAND, P.C. (1974). Hormones profiles in breast disease. M.D. Thesis, University of Manchester.

ERNST, M. (1925). Die physiologischen rückbildungeserscheinungen in der wieblichen brustdrüse nach gravidität und menstruation. *Frankfurter Zeitschrift für Pathologie,* 31, 500-506.

ERNSTER, V.L., SACKS, S.J. PETERSON, C.A. & SCHWEITZER, R.J. (1980). Mammographic parenchymal patterns and risk factors for breast cancer. *Radiology,* 134, 617-620.

EVARTS, R.P. & BROWN, C.A. (1977). Morphology of mammary glands, ovaries and pituitary gland of hydroxylamine-fed C3H/HeN mice. *Laboratory Investigation,* 37, 53-63.

FANGER, H. & REE, H.J. (1974). Cyclic changes of human mammary gland epithelium in relation to the menstrual cycle - an ultrastructural study. *Cancer,* 34 571-585.

FERGUSON, D.J.P. & ANDERSON, T.J. (1981). Morphological evaluation of cell turnover in relation to the menstrual cycle in the resting human breast. *British Journal of Cancer*, 44, 177-181.

FIKETE, E. (1938). A comparative morphological study of the mammary gland in a high and low tumour strain of mice. *American Journal of Pathology*, 14, 557-578.

FISHER, E.R., DOW, W.A. & POSADA, H. (1973). Correlations between specimen roentgenograms (mammograms) and pathologic findings in breast disease. In: *Pathology Annual, edited by Sommers, S.C.* pp. 453-472. Appleton-Century Crofts, New York.

FISHER, E.R., POSADA,H. & RAMOS, H. (1974). Evaluation of mammography based upon correlation of specimen mammograms and histopathologic findings. *American Journal of Clinical Pathology*, 62, 60-72.

FISHER, E.R., PALEKAR, A., KIM, W.S. & REDMOND, C. (1978). The histopathology of mammographic patterns. *American Journal of Clinical Pathology*, 69, 421-426.

FITTS, W.T. Jr. & DONALD, J.G. (1949). Diagnosis of lesions of the breast. *Surgery*, 25, 424-430.

FOLLEY, S.J., GUTHKELCH, A.N. & ZUCKERMAN, S. (1939). The mammary gland of the rhesus monkey under normal and experimental conditions. *Proceedings of the Royal Society. Series B. Biological Sciences*, 126, 469-491.

FOOTE, F.W. & STEWART, F.W. (1945a). Comparative studies of cancerous versus non cancerous breasts. 1. Basic morphologic characteristics. *Annals of Surgery*, 121, 6-53.

FOOTE, F.W. & STEWART, F.W. (1945b). Comparative studies of cancerous versus non cancerous breasts. II. Role of so-called chronic cystic mastitis in mammary carcinogenesis. Influence of certain hormones on human breast structure. *Annals of Surgery,* 121, 197-222.

FLUX, D.S. (1954a). Growth of the mammary duct system in the intact ovariectomized mice of the CHI strain. *Journal of Endocrinology,* 11, 223-237.

FLUX, D.S. (1954b). The effect of adrenal steroids on the growth of the mammary glands, uteri, thymus and adrenal glands of intact ovariectomized and oestrone-treated ovariectomized mice. *Journal of Endocrinology,* 11, 238-254.

FRANK, R.T. (1931). The hormonal causes of premenstrual tension. *Archives of Neurology and Psychiatry,* 26, 1053-1057.

FRANTZ, V.K., PICKREN, J.W., MELCHER, G.W. & AUCHINCLOSS, H. Jr. (1951). Incidence of chronic cystic disease in so-called normal breasts. A study based on 225 postmortem examinations. *Cancer,* 4, 762-783.

FRAY, W.W., & WARREN, S.L. (1932). Stereoscopic roentgenography of breasts, an aid in establishing diagnosis of mastitis and carcinoma. *Annals of Surgery,*95, 425-432.

GABRILOVE, J.L. & LUDWIG, A.W. (1957). The histogenesis of myxedema. *Journal of Clinical Endocrinology and Metabolism,* 17, 925 - 932.

GALKIN, B.M., FRASCA, P., FEIG, S.A. & HOLDERNESS, K.E. (1982). Non-calcified breast particles. A possible new marker of breast cancer. *Investigative Radiology,* 17, 119-128.

GALLAGER, H.S. & MARTIN, J.E. (1969). Early phases in the development of breast cancer. *Cancer,* 24, 1170-1178.

GARDNER, W.U. (1935). The effect of ovarian hormones and ovarian grafts upon the mammary glands of male mice. *Endocrinology,* 19, 656-667.

GARDNER, W.U., DIDDLE, A.W., ALLEN, E. & STRONG, L.C. (1934). The effect of theelin on the mammary rudiments of male mice differing in susceptibility to tumour development. *Anatomical Record,* 60, 457-476.

GARDNER, W.U. & STRONG, L.C. (1935). The normal development of the mammary glands of virgin female mice of ten strains varying in susceptibility to spontaneous neoplasms. *American Journal of Cancer,* 25, 282-290.

GARDNER, W.U. & VAN WAGENEN, G. (1938). Experimental development of the mammary gland of the monkey. *Endocrinology,* 22, 164-172.

GARFINKEL, L. CRAIG, L. & SEIDMAN, H. (1959). An appraisal of left and right breast cancer. *Journal of the National Cancer Institute,* 23, 617-631.

GERSCH, I. & CATCHPOLE, H.R. (1960). The nature of ground substance of connective tissue. *Perspectives in Biology and Medicine,* 3, 282-319.

GESCHICKTER, C.F. (1945). Diseases of the Breast: Diagnosis, Pathology, Treatment. J.B. Lippincott Co., Philadelphia, London.

GILLMAN, J. (1940). Experimental studies on the menstrual cycle of the baboon, *(papio porcaris)*VI. The effect of progesterone upon the first part of the cycle in normal female baboons. *Endocrinology,* 26, 80-87.

GIVENS, J.R., ANDERSON, R.N., RAGLAND, J.B., WISER, W.L. & UNSTOT, E.S. (1975). Adrenal function in hirsutism. 1. Diurnal change and response of plasma androstenedione, testosterone, 17-hydroxyprogesterone, cortisol, LH and FSH to dexamethasone and ½ unit of ACTH. *Journal of Clinical Endocrinology and Metabolism*, 40, 988-1000.

GOING, J. (1977). The histochemical nature of the mucins and the fine structure of pulmonary adenocarcinomas and bronchiolo-alveolar carcinomas. B.Sc. Thesis, University of Edinburgh.

GOLINGER, R.C. (1978). Hormones and the pathophysiology of fibrocystic mastopathy. *Surgery, Gynecology and Obstetrics*, 146, 273-285.

GOUGH, J. & WENTWORTH, J.E. (1949). The pathology of the pneumoconiosis in Wales. A new technique for the study of lung pathology. *Proceedings of the Ninth International Congress on Industrial Medicine*. pp. 661-666. John Wright and Sons Ltd., Bristol.

GRAVELLE, I.H., BULSTRODE, J.C., WANG, D.Y., BULBROOK, R.D.& HAYWARD, J.L. (1980). The relation between radiographic features and determinants of risk of breast cancer. *British Journal of Radiology*, 53, 107-113.

GRISHMAN, E. (1952). Histochemical analysis of muco-polysaccharides occurring in mucus-producing tumors; mixed tumors of parotid gland, colloid carcinomas of breast and myxomas. *Cancer*, 5, 700-707.

GROSSFELD, H., MEYER, K. & GODMAN, G. (1955). Differentiation of fibroblasts in tissue culture, as determined by mucopolysaccharide production. *Proceedings of the Society for Experimental Biology and Medicine*, 88, 31-35.

HAAGENSEN, C.D. (1971). Diseases of the Breast. 2nd Ed. Holt-Saunders Ltd., Philadelphia.

HADDOCK, N.H. (1947). Alcian blue, a new phthalocyanine dyestuff. *Research,* 1, 685-689.

HAINLINE, S., MYER, L., McLELLAND, R., NEWELL, J., GRUFFERMAN, S. & SHINGLETON, W. (1978). Mammographic patterns and risk of breast cancer. *American Journal of Roentgenology,* 130, 1157-1158.

HAMILTON, T. & RANKIN, M.E. (1975). Changes in volume of the breast during the menstrual cycle. *British Journal of Surgery,* 62, 660.

HARDESTY, M. (1931). The structural basis for the response of the comb of the brown leghorn fowl to the sex hormones. *American Journal of Anatomy,* 47, 277-325.

HARNETT, W.L. (1948). A statistical report on 2529 cases of cancer of the breast. *British Journal of Cancer,* 12, 212-239.

HERTZLER, A.E. (1933). Surgical Pathology of the Mammary Gland. p. 36. J.B. Lippincott Co., Philadelphia.

HICKEN, N.F. (1937). Mammography. The roentgenographic diagnosis of breast tumours by means of contrast media. *Surgery, Gynecology and Obstetrics,* 64, 593-603.

HICKEN, N.F., (1940). Mastectomy. A clinical pathologic study demonstrating why most mastectomies result in incomplete removal of the mammary gland. *Archives of Surgery,* 40, 6-14.

HICKEN, N.F., BEST, R., HUNT, H.B. & HARRIS, T.T. (1938). The roentgen visualisation and diagnosis of breast lesions by means of contrast media. *American Journal of Roentgenology,* 39, 321-343.

HIROHATA, T., NOMURA, A.M.Y.& KOLONEL, L.N. (1977). Breast size and cancer. *British Medical Journal*, 2, 641.

HITSCHMANN, F.& ADLER, L. (1908). Der bau der uterusschleimhaut des geschlechtsreifen weibes mit besönderer berucksichtigung der menstruation. *Monatsschrift für geburtshilfe u. Gynäkologie*, 27, 1-82.

HORTON, R.E. (1945). Erosional development of streams and their drainage basins: hydrophysical approach to quantitative morphology. *Bulletin of the Geological Society of America*, 56, 275-370.

HUMPREY, L.T. & SWERDLOW, H. (1966). Histologic changes in clinically normal breasts at postmorten examination. *Archives of Surgery*, 92, 192-193.

HUSEBY, R.A. & BITTNER, J.J. (1946). A comparative morphological study of the mammary glands with reference to the known factors influencing the development of mammary carcinoma in mice. *Cancer Research*, 6, 240-255.

HUSEBY, R.A. & THOMAS, L.B., (1954). Histological and histochemical alterations in the normal breast tissues of patients with advanced cancer being treated with estrogenic hormones. *Cancer*, 7, 54-74.

HUTTER, R.V.P.& KIM, D.V. (1971). The problem of multiple lesions of the breast. *Cancer*, 28, 1591-1607.

HVIDBERG, E. & JENSEN, C.E. (1959). Changes in molecular weight of acid mucopolysaccharides in connective tissue due to hormone treatment, dehydration and age. *Acta Chemica Scandinavica*, 13, 2047-2056.

IHNEN, M. & PEREZ-TAMAYO, R. (1953). Breast stroma. Morphological and histochemical study. *Archives of Pathology,* 56, 46-67.

INGLEBY, H. (1932). Relation of fibro-adenoma and chronic mastitis to sexual cycle changes in the breast. *Archives of Pathology,* 14, 21-41.

INGLEBY, H. (1949). Changes in breast volume in a group of normal young women. *Bulletin of the International Association of Medical Museums,* 29, 87-94.

INGLEBY, H & HOLLY, C. (1939). A method for the preparation of serial slices of the breast. *Bulletion of the International Association of Medical Museums,* 19, 93-96.

INGLEBY, H. & GERSHON-COHEN, J. (1960). Comparative Anatomy, Pathology and Roentgenology of the Breast. University of Philadelphia Press, Philadelphia.

ISARD, H.J.& SHILO, R. (1968). Breast thermography. *American Journal of Roentgenology,* 103, 921-925.

ISRAEL, S.L. (1938). Premenstrual tension. *Journal of the American Medical Association,* 110, 1721-1723.

ISRAEL, S.L. (1967). Diagnosis and Treatment of Menstrual Disorders and Sterility. 5th Ed. p. 154. Harper & Row Publishers, New York, Evanston, London.

IVERSON, O.H. (1960). Isolation of acid mucopolysaccharides in the myometrium of the human cervix uteri. *Acta Pathologica et Microbiologica Scandinavica,* 50, 25-28.

JACOBSON, B. (1978). Hyaluronic acid synthesis in rooster comb. Effect of testosterone on nucleotide sugar metabolism. *Connective Tissue Research,* 5, 217-223.

JENSEN, C.E. & ZACHARIAE, F. (1958). Studies on the mechanism of ovulation. Isolation and analysis of acid mucopolysaccharides in bovine follicular fluid. *Acta Endocrinologica*, 27, 356-368.

JENSEN, H.M., RICE, J.R. & WELLINGS, S.R. (1976). Preneoplastic lesions in the human breast. *Science*, 191, 295-297.

JENSEN, H.M. & WELLINGS, S.R. (1976). Preneoplastic lesions of the human mammary gland transplanted into the nude athymic mouse. *Cancer Research*, 36, 2605-2610.

JONES, C.H. (1982). Methods of breast imaging. *Physics in Medicine and Biology*, 27, 463-499.

JUDD, H.L. & YEN, S.S.C. (1973). Serum androstenedione and testosterone levels during the menstrual cycle. *Journal of Clinical Endocrinology and Metabolism*, 36, 475-481.

KALACHE, A. (1981). Risk factors for breast cancer: a tabular summary of the epidemiological literature. *British Journal of Surgery*, 68, 797-799.

KATARIYA, R.N., FORREST, A.P.M. & GRAVELLE, I.H. (1974). Breast volumes in cancer of the breast. *British Journal of Cancer*, 29, 270-273.

KHANOLKAR, V.R. & RANADIVE, K.J. (1947). The effect of foster nursing on the morphology of the mammary gland in mice. *Journal of Pathology and Bacteriology*, 59, 593-603.

KLEMPERER, P., POLLACK, A.D. & BAEHR, G. (1941). Pathology of disseminated lupus erythematosus. *Archives of Pathology*, 32, 569-631.

KNOBEL, D.P. (1966). The development of the mammary glands in the mouse. Ph.D. Thesis, University of of Edinburgh.

KOLGANOVA, I.P. & ZOLOTAREVSKY, I.P. (1981). Radioanatomic correlations in the study of the intact mammary gland. *Vestnik Rentgenologii i Radiologii*, pp. 65-72.

KORIBA, K. (1958). On the periodicity of tree growth in the tropics. *Singapore Garden Bulletin*, 17, 11-81.

KRAMER, W.M. & RUSH, B.F. Jr. (1973). Mammary duct proliferation in the elderly: a histopathologic study. *Cancer*, 31, 130-137.

KROOK, P.M. (1978). Mammographic parenchymal patterns as risk indicators for incident cancer in a screening program: an extended study. *American Journal of Roentgenology*, 131, 1031-1035.

KROOK, P.M., CARLILE, T., BUSH, W. & HALL, M.H. (1978). Mammographic parenchymal patterns as a risk indicator for prevalent and incident cancer. *Cancer*, 41, 1093-1097.

KURU, H. (1909). Beiträge zur geschwulstlehre. *Deutsche Zeitschrift für Chirurgie*, 98, 414-463.

LAMB, D. (1968). Intracellular development and secretion of mucus in the normal and morbid bronchial tree. Ph.D. Thesis, University of London.

LANDAU, R.L., BERGENSTAHL, D.M., LUGIBIHI, K. & KASCHT, M.E. (1955). The metabolic effects of progesterone in man. *Journal of Clinical Endocrinology and Metabolism*, 15, 1194-1215.

LANE-CLAYPON, J.E. (1924). Cancer of the breast and its surgical treatment. *Reports on Public Health and Medical Subjects, No.* 28, p. 109, H.M.S.O., London.

LANE-CLAYPON, J.E. (1926). A further report on cancer of the breast with special reference to its

associated antecedent conditions. *Reports on Public Health and Medical Subjects, No.* 32, pp. 75-80. H.M.S.O., London.

LANE-CLAYPON, J.E. (1928). Report on the late results of operation for cancer of the breast. *Reports on Public Health and Medical Subjects, No.* 51, p.11, H.M.S.O., London.

LAURENCE, D.J.R. (1978). Milk proteins and mammary cancer: a review. *Investigative and Cell Pathology,* 1, 5-22.

LAURENT, T.C. (1964). The interaction between polysaccharides and other macromolecules. 9. The exclusion of molecules from hyaluronic acid gels and solutions. *Biochemical Journal,* 93, 106-112.

LAURENT, T.C., BJÖRK, I., PIETRUSZKIEWICZ, A. & PERSSON, H. (1963). On the interaction between polysaccharides and other macromolecules. *Biochimica et Biophysica Acta,* 78, 351-336.

LAURENT, T.C. & OGSTON, A.G. (1963). The interaction between polysaccharides and other macromolecules. 4. The osmotic pressure of mixtures of serum albumin and hyaluronic acid. *Biochemical Journal,* 89, 249-253.

LAURENT, T.C. & KILLANDER, J. (1964). Theory of gel filtration and its experimental verification *Journal of Chromatography,* 14, 317-330.

LEBORGNE, R. (1953). The Breast in Roentgen Diagnosis. p. 21. Impressora Uraguaya S.A., Montivideo.

Le CORBUSIER. (1971). Architecture and the mathematical spirit. In: *Great Currents of Mathematical Thought, Vol. II, edited by Le Lionnais, F.* pp. 174-188. Dover Publications Inc., New York.

LEIS, H.P.Jr.(1978). Epidemiology of breast cancer: identification of the high risk women. In: *The Breast, edited by Gallager, H.S., Leis, H.P. Jr., Snyderman, R.K., Urban, J.A.* pp. 37-48. The C.V. Mosby Company, Saint Louis.

LEUSCHNER, U. (1969). Über die lokalisation von mucopolysacchariden und mastzellen in scirrhösen carcinomen der mamma. *Acta Histochemica,* 34, 126-137.

LEV, R. & SPICER, S.S. (1964). Specific staining of sulphate groups with alcian blue at low pH. *Journal of Histochemistry and Cytochemistry,* 12, 309.

LIKAR, L.J., MASON, M.M. & ROSENKRANTZ, H. (1963). Response of the level of acid mucopolysaccharides in rat granulation tissue to cortisol. *Endocrinology,* 72, 393-396.

LILLIE, R.D. (1965). Histopathologic Technic and Practical Histochemistry. 3rd Ed. p. 539. McGraw-Hill Book Company, New York, London.

LOBOTSKY, J., WYSS, H.I., SEGRE, E.J. & LLOYD, C.W. (1964). Plasma testosterone in the normal woman. *Journal of Clinical Endocrinology and Metabolism,* 24, 1261-1265.

LOCKWOOD, I.H. (1934). The value of breast radiography. *Radiology,* 23, 202-207.

LOCKWOOD, I.H. & STEWART, W. (1932). Roentgen study of physiologic and pathologic changes in the mammary gland. *Journal of the American Medical Association,* 99, 1461-1466.

LOESCHKE, H. (1925). Ueber zyklische vorgänge in den drüsen des achselhöhlenorgans und ihre abhängigkeit vom sexualzyklus des weibes. *Virchows Archiv für pathologische Anatomie und Physiologie und für klinische Medizin,* 255, 283-294.

LUCHSINGER y CENTENO, J. (1927). Üeber die cyklischen veränderungen der weiblichen brustdrüse. *Beiträge zur pathologischen Anatomie und zur allgemeinen Pathologie*, 78, 594-617.

LURIE, M.B. & ZAPPASODI, P. (1942). Effect of chorionic gonadotropin on spread of particulate substances in skin of rabbits. *Archives of Pathology*, 34, 151-166.

McGILVERY, R.W. (1979). Biochemistry. A Functional Approach. 2nd Ed. p. 186. W.B. Saunders Co. Ltd., London.

McGREGOR, A.L. & Du PLESSIS, D.J. (1969). A Synopsis of Surgical Anatomy. 10th Ed. p. 34. John Wright and Sons Ltd., Bristol.

McKAY, D.G. (1950). Metachromasia in the endometrium. *American Journal of Obstetrics and Gynecology*, 59, 875-882.

McMAHON, B., COLE, P. & BROWN, J. (1973). The etiology of human breast cancer: a review. *Journal of the National Cancer Institute*, 50, 21-42.

MANCINI, R.E., BUR, G., BRANDES, D. & GARBERI, J.C. (1951). Mucoproteinas de la sangre y del tejido conectivo de fibroadenoma mamario. *Revista de la Sociedao Argentina de Biologia*, 27, 243-251.

MANNOZZI-TORINI, M. (1942). Comportamento dei granuli delle cellule basofile connettivali dopo azione dell'enzima mucinolitico testicolare. *Bollettino della Societa Italiana di Biologia Sperimentale*, 17, 153.

MANTON, S.L., FERGUSON, D.J.P. & ANDERSON, T.J. (1981). An automated technique for the rapid processing of breast tissue for subgross examination. *Journal of Clinical Pathology*, 34, 1189-1191.

MARCHANT, D.A. (1979). Epidemiology of breast cancer. In: *Breast Disease. Proceedings of an International Symposium, May 13-17, 1978, edited by Marchant, D.A., Nyirjesy, I.* pp. 54-61. Grune and Stratton Inc., New York, London.

MARCUM, R.G. & WELLINGS, S.R. (1969). Subgross pathology of the human breast: method and initial observations. *Journal of the National Cancer Institute,* 42, 115-121.

MATTHEWS, R.W. (1973). Structural and functional studies of the acini and granular convoluted tubule in the submandibular gland of *rattus norwegicus*. Ph.D. Thesis, University of Newcastle.

MAXWELL, A.E. (1967). Analysing Quantitative Data. p. 115. Methuen & Co. Ltd., London.

MAYHEW, T.M. & WILLIAMS M.A. (1971). A comparison of two sampling procedures for stereological analysis of cell pellets. *Journal of Microscopy,* 94, 195-204.

MEINHARD, E.A. (1974). Histoquantitation using computer data cards. *Journal of Microscopy,* 101, 95-102.

MEINHARD, E.A., WADBROOK D.G. & RISDON, R.A. (1975). Computer card morphometry of jejunal biopsies in childhood coeliac disease. *Journal of Clinical Pathology,* 28, 85-93.

MENDELL, L., ROSENBLOOM, M.& NAIMARK, A. (1977). Are breast patterns a risk index for breast cancer? A reappraisal. *American Journal of Roentgenology,* 128, 547.

MERIGGI, A., AZZINI, N. & CHIESA, A. (1964). Ricerche istochimiche sulla mammella sana e normale di donne in giovane eta. *Ospedale Maggiore,* 59, 1344-1350.

MEYER, K. & PALMER,J.W. (1934). Polysaccharide of vitreous humor. *Journal of Biological Chemistry,* 107, 629-634.

MEYER, K. & RAPPORT M.M. (1951). The mucopolysaccharides of the ground substance of connective tissue. *Science*, 113, 596-599.

MEYER, K., DAVIDSON, E., LINKER, A. & HOFFMAN, P. (1956). The acid mucopolysaccharides of connective tissue. *Biochimica et Biophysica Acta*, 21, 506-518.

MEYER, K., HOFFMAN, P. & LINKER, A. (1957).The acid mucopolysaccharides of connective tissue. In: *Connective Tissue, edited by Tunbridge, R.E., Madeline, K., Delafresnaye, J.F., Wood, G.C.* pp. 86-96. Blackwell Scientific Publications, Oxford.

MIGLIORI, E. (1975). Un metodo di studio submacroscopico della mammella umana intera. *Tumori*, 61, 357-363.

MILLER, A.B. (1980). Breast cancer etiologic influences. In: *Breast Cancer: New Concepts in Etiology and Control, edited by Brennan, M.J., McGrath, C.M., Rich, M.A.* pp. 17-27. Academic Press Inc., New York, London.

MILLIGAN, D., DRIFE, J.O. & SHORT, R.V. (1975). Changes in breast volume during normal menstrual cycle and after oral contraceptives. *British Medical Journal*, 4, 494-496.

MONTAGU, M.F.A. (1960). An Introduction to Physical Anthropology. 3rd Ed. p. 579. Charles C. Thomas, Springfield, Illinois.

MORTON, J.H., ADDITON, H. ADDISON, R.G., HUNT, L. & SULLIVAN J.J. (1953). A clinical study of premenstrual tension. *American Journal of Obstetrics and Gynecology*, 65, 1182-1191.

MOSZKOWICZ, L. (1926). Üeber den monatlichen zyklus der brustdrüse. *Archiv für klinische Chirurgie*, 142, 374-418.

MOSKOWITZ, M., GARTSIDE, P.& McLAUGHLIN, C. (1980). Mammographic patterns as markers for high risk benign breast disease and incident cancers. *Radiology,* 134, 293-295.

MOSTELLER, F. & ROURKE, R.E.K. (1973). Sturdy Statistics. Nonparametric and Order Statistics. Addison-Wesley Publishing Co., Reading, Massachusetts, London.

MOWRY, R.W. (1956). Alcian blue techniques for the histochemical study of acidic carbohydrates. *Journal of Histochemistry and Cytochemistry,* 4, 407.

MUIR, H. (1964). Chemistry and metabolism of connective tissue glycosaminoglycans (mucopolysaccharides). *International Review of Connective Tissue Research,* 2, 101-154.

MUNFORD, R.E. (1964). A review of anatomical and biochemical changes in the mammary gland with particular reference to quantitative methods of assessing mammary development. *Dairy Science Abstracts,* 26, 293-304.

MURRAY, C.D. (1926). The physiological principle of minimum work applied to the angle of branching of arteries. *Journal of General Physiology,* 9, 835-841.

MURRAY, C.D. (1927). A relationship between circumference and weight in trees and its bearing on branching angles. *Journal of General Physiology,* 10, 725-739.

MYERS, J.A. (1916). Studies on the mammary gland. 1. The growth and distribution of the milk ducts and the development of the nipple in the albino rat from birth to ten weeks of age. *American Journal of Anatomy,* 19, 353-390.

NEWTON, M. (1961). Human lactaction. In: *Milk: The Mammary Gland and its Secretion, Vol. I, edited by Kon, S.K., Cowie, A.T.* pp. 281-320. Academic Press Inc., New York, London.

NICOLSON, W.P. Jr. & GRADY, E.D. (1948). Carcinoma of the breast. *Annals of Surgery,* 127, 992-1009.

NIENHAUS, H. & BRENNER, M. (1977). Morphometrie des brustdrüsengewebes. *Microscopica Acta Supplement,* 1, 113-119.

NIZZE, H. (1972). Zur biomorphose des mantelbindegewebes der weiblichen brustdrüse. *Virchows Archiv. Abteilung A. Pathologie und pathologiesche Anatomie,* 356, 249-258.

OLIVI, M & BARBIERI, G. (1952). La mastopathia fibrocistica. Ricerca sistematica sulca metachromasia stromale e sulle mastzellen nella mastopatia fibrocistica, con riferimento alla mammella normale e al carcinoma mammario. *Lavori dell Istituto di Anatomia e Istologia Patologica,* 12, 311-323.

OZZELLO, L. & SPEER, F.D. (1958). The mucopolysaccharides in the normal and diseased breast. *American Journal of Pathology,* 34, 993-1009.

PALLOT, G. (1935). Recherches histologiques sur la mamelle prémenstruelle. *Bulletin d'histologie appliquée à la physiologie et à la pathologie et de technique microscopique,* 12, 378-399.

PAPANICOLAU, G.N., HOLMQVIST, D.G., BADER, C.M. & FALK E.A. (1958). Exfoliative cytology of the human mammary gland and its value in the diagnosis of cancer and other diseases of the breast. *Cancer,* 11, 377-409.

PARBOOSINGH, J., DOIG, A. & MICHIE, E.A. (1973). Renal excretion of water and solutes during the normal menstrual cycle. *Journal of Obstetrics and Gynaecology of the British Commonwealth,* 80, 978-983.

PARKS, A.G. (1959). The micro-anatomy of the breast. *Annals of the Royal College of Surgeons of England*, 25, 235-251.

PARRY, C.E., FREUNDLICH, I.M. & WALLACE, J.D. (1972). Breast thermograms in ovulatory and anovulatory menstrual cycles. *British Journal of Radiology*, 45, 507-509.

PAYNE, K.T. & HUTCHINS, G.M. (1973). Rapid production of reconstruction drawings from serial sections. *American Journal of Clinical Pathology*, 60, 820-822.

PETRAKIS, N.L. (1977). Breast secretory activity in non-lactating women, post-partum breast involution, and the epidemiology of breast cancer. *National Cancer Institute Monograph*, 47, 161-164.

PETRAKIS, N.L., MASON, L., LEE, R., SUGIMOTO, B., DAWSON, S. & CATCHPOOL, F. (1975). Association of race, age, menopausal status and cerumen type with breast secretion in non-lactating women, as determined by nipple aspiration. *Journal of the National Cancer Institute*, 54, 829-834.

PEYSTER, R.G., KALISHER, L. & COLE, P. (1977). Mammographic parenchymal patterns and the prevalence of breast cancer. *Radiology*, 125, 387-391.

PHILIP, J., HARRIS, W.G. & RUSTAGE, J.H. (1982). Radiographs of breast biopsy specimens. *British Journal of Surgery*, 69, 126-127.

PICKLES, V.R. (1953). Blood-flow estimations as indices of mammary activity. *Journal of Obstetrics and Gynaecology of the British Empire*, 60, 301-311.

POLANO, R. (1924). Untersuchungen über die zyklischen veränderungen der weiblichen brust während der geschlechtsreife. *Zeitschrift für Geburtshilfe und Gynäkologie*, 87, 363-373.

POZZI, P.C., MERIGGI, A. & DAGRADA, T. (1964). Studio istochimico della mammella normale di donna giovane mediante la reazione alcian-bleu a vari pH. *Biologica latina,* 17, 517-522.

PREECE, P.E., HUGHES, L.E., BAUM, M.& RICHARDS, A.R. (1974). Studies on breast pain. *British Journal of Surgery,* 61, 322.

PRIEST, R.E. (1961). Hormonal control of acid mucopolysaccharide synthesis. In: *Hahnemann Symposium on Inflammation and Diseases of Connective Tissue, edited by Mills, L.C., Moyer, J.H.* pp. 44-46. W.B. Saunders Co. Ltd., Philadelphia.

QUINTARELLI, G., TSUIKI, S., HASHIMOTO, Y. & PIGMAN, W. (1960). Histochemical studies of bovine salivary gland mucins. *Biochemical and Biophysical Research Communications,* 2, 423-426.

REHMAN, I. (1978). Embryology and anatomy of the breast. In: *The Breast, edited by Gallager, H.S., Leis, H.P. Jr., Snyderman, R.K., Urban, J.A.* pp. 3-22. The C.V. Mosby Company, Saint Louis.

REIMANN, S.P. & SEABOLD, P.S. (1933). Correlation of X-ray picture with histology in certain breast lesions. *American Journal of Cancer,* 17, 34-41.

RICHARDS, F.J. & SCHWABE, W.W. (1969). Phyllotaxis: a problem of growth and form. In: *Plant Physiology. A Treatise, Vol. VA, edited by Steward, F.C.* pp. 79-116. Academic Press Inc., New York, London.

RICHARDSON, K.C. (1947). Some structural features of the mammary tissues. *British Medical Bulletin,* 5, 123-129.

RICHARDSON, K.C. (1952). The measurement of lactating mammary epithelium. *Journal of Anatomy,* 86, 472.

RIDEOUT, D.F. & POON, P.Y. (1977). Patterns of breast parenchyma on mammography. *Journal of the Canadian Association of Radiologists,* 28, 257-258.

RISDON, R.A. (1974). Quantitation of histological changes in the glomeruli in renal biopsy specimens from children: total and differential glomerular cell counts. *Journal of Pathology,* 114, 185-197.

ROSENBURG, A. (1922). Üeber menstruelle durch das corpus luteum bedingte mammaveränderungen. *Frankfurter zeitschrift für Pathologie,* 27, 466-506.

ROSENBURG, A. (1923). Die bedeutung der menstruellen mammaveränderungen für die chirurgie. *Zentralblatt für Chirurgie,* 1, 510.

ROSS, G.T., CARGILLE, C.M., LIPSETT, M.B., RAYFORD, P.L., MARSHALL, J.R., STROTT, C.A. & RODBARD, D. (1970). Pituitary and gonadal hormones in women during spontaneous and induced ovulatory cycles. *Recent Progress in Hormone Research,* 26, 1-62.

RUSH, B.F. Jr. (1974). Breast. In: *Principles of Surgery. 2nd Ed. Editor in Chief, Schwartz, S.I.* pp. 527-554. McGraw-Hill Book Co., New York, London.

RUSSO, J. & RUSSO, I.H. (1978) DNA labelling index and structure of the rat mammary gland as determinants of its susceptibility to carcinogenesis. *Journal of the National Cancer Institute,* 61, 1451-1460.

SALK, L. (1970). The critical nature of the postpartum period in the human for the establishment of the mother-infant bond: a controlled study. *Diseases of the Nervous System,* 31, 110-116.

SALOMON, A. (1913). Beitrage zur pathologie und klinik das mammakarzinoms. *Archiv für klinische Chirurgie,* 101, 573-668.

SANDISON, A.T. (1962). A post-morten survey of the breast. *National Cancer Institute Monograph,* 8, 1-145.

SANDISON, A.T. & WALKER,J.C. (1968). Diseases of the adolescent female breast. *British Journal of Surgery,* 55, 443-448.

SARNELLI, R., SABÒ, C & SQUARTINI, F. (1980a). Subgross physiopathology of the breast associated with clinical cancer. *Tumori,* 66, 565-582.

SARNELLI, R,, ORLANDI, F., MIGLIORI, E.& SQUARTINI,F. (1980b). Morfologica submacroscopica della mammella: reperti semeiologici, nomenclatura e possibilità di applicazione del metodo. *Pathologica,* 72, 139-187.

SASANO, N., TATENO, H.& STEMMERMANN, G.N. (1978). Volume and hyperplastic lesions of breasts of Japanese women in Hawaii and Japan. *Preventive Medicine,* 7, 196-204.

SCHIFF, M. (1966). The influence of estrogens on connective tissue. In: *Hormones and Connective Tissue, edited by Asboe-Hansen, G.* p. 287. Munksgaard, Copenhagen.

SCHILLER, S., BENDITT, E.P. & DORFMAN, A. (1952). Effect of testosterone and cortisone on the hexosamine content and metachromasia of chick combs. *Endocrinology,* 50, 504-510.

SCHWARTZ. S.R. & SOONEUNG, K. (1969). An improved method for the preparation of whole organ mounts for light microscopy. Localisation of calcium salts and corrective radiographic technics using whole breasts. *American Journal of Clinical Pathology,* 51, 511-515.

SCOMMEGNA, A., VORYS, N. & GIVENS, J.R. (1980). Menstrual dysfunction. In: *Gynecologic Endocrinology, 3rd Ed., edited by Gold, J.J., Josimovich, J.B.* pp. 290-326. Harper and Row Publishers, Hagerstown.

SEBENING, W. (1925). Zur physiologie und pathologie der brustdrüse. (Die menstruellen veränderungen der weiblichen brustdrüse. Das krankheitsbild der schmerzhaften knötenbildung. Mastitis chronica cystica). *Archiv für klinische Chirurgie,* 134, 464-485.

SENIE, R.T., ROSEN, P.P., LESSER, M.L., SNYDER, R.E., SCHOTTENFELD, D. & DUTHIE, K. (1980). Epidemiology of breast carcinoma. II. Factors related to the predominance of left-sided disease. *Cancer,* 46, 1705-1713.

SHORT, R.H.D. (1950). Alveolar epithelium in relation to growth of the lung. *Philosophical Transactions of the Royal Society of London,* 235, 35-86.

SHORT, R.H.D. (1952). Aspects of comparative lung growth. *Proceedings of the Royal Society of London. Series B. Biological Sciences,* 140, 432-441.

SHORT, R.V. (1974). Man, the changing animal. In: *Physiology and Genetics of Reproduction. Part A, edited by Coutinho, E.M., Fuchs, F.* pp. 3-15. Plenum Press, New York, London.

SHORT, R.V. & DRIFE, J.O. (1977). The aetiology of mammary cancer in man and animals. *Symposia of the Zoological Society of London,* 41, 211-230.

SIEGEL, S. (1956). Nonparametric Statistics for the Behavioral Sciences. p. 116. McGraw-Hill, Kogakusha Ltd., Tokyo.

SIEMENS, H.W. (1952). Über die form der weiblichen brust, insonderheit den descensus mammae. *Virchows Archiv für pathologische Anatomie und Physiologie und für klinische Medizin,* 322, 101-118.

SILVER, M. (1953a). A quantitative analysis of the role of oestrogen in mammary development in the rat. *Journal of Endocrinology,* 10, 17-34.

SILVER, M. (1953b). The onset of allometric mammary growth in the female hooded norway rat. *Journal of Endocrinology,* 10, 35-45.

SIMKIN, B & GOODART, D. (1960). Preliminary observations on prolactin activity in human blood. *Journal of Clinical Endocrinology and Metabolism,* 20, 1095-1106.

SLOSS, P.T., BENNETT, W.A. & CLAGETT, O.T. (1957). Incidence in normal breasts of features associated with chronic cystic mastitis. *American Journal of Pathology,* 33, 1181-1192.

SMALL, E.C. (1979). A breast conscious society. In: *Breast Disease. Proceedings of an International Symposium, May 13-17, 1978, edited by Marchant, D.A., Nyirjesy, I.* pp. 1-8. New York, San Fransisco, London.

SMITHERS, D.W., RIGBY-JONES, P., GALTON, D.A.G. & PAYNE, P.M. (1952). Cancer of the breast. A review. *British Journal of Radiology. Supplement,* 4, 1-90.

SOINI, I. (1977). Risk factors of breast cancer in Finland. *International Journal of Epidemiology,* 6, 365-373.

SPEROFF, L. & VANDE WIELE, R.L. (1971). Regulation of the human menstrual cycle. *American Journal of Obstetrics and Gynecology,* 109, 234-247.

SPEROFF, L., GLASS, R.H. & KASE, N.G. (1978). Clinical Gynecologic Endocrinology and Infertility. 2nd Ed. p. 58. Williams and Wilins Co., Baltimore.

STACEY, M. & BARKER, S.A. (1962). Carbohydrates of Living Tissue. p. IX. D. Van Nostrand Company Ltd., London.

STEEDMAN, H.F. (1950). Alcian blue 8GS: a new stain for mucin. *Quarterly Journal of Microscopical Science,* 91, 477-479.

STEVENS, M. (1978). Traumatic breast cancer. *Medical Trial Technique Quarterly,* 25, 1-9.

STEVENS, P.S. (1976). Patterns in Nature. Penguin Books Ltd., Harmondsworth, England.

STILES, H.J. (1892). Contributions to the surgical anatomy of the breast: with a note of the "nitric acid method" of studying its normal and pathological anatomy. *Edinburgh Medical Journal,* 37, 1099-1112.

STRYER, L. (1981). Biochemistry. 2nd Ed. p. 200. W.H. Freeman and Co., San Fransisco.

SYLVÉN, B. (1938). Über die elektivität und fehlerquellen der schleimfärbung mit mucicarmin im vergleich mit metachromatischer färbung. *Virchows Archiv für pathologische Anatomie und Physiologie und für klinische Medizin,* 303, 280-394.

SYLVÉN, B. (1945). Ester sulphuric acids of high molecular weight and mast cells in mesenchymal tumours. Histochemical studies on tumorous growth. *Acta Radiologica Supplementum,* 59, 1-99.

SYRÉ, G. & SEHN, M. (1981). Intracellular storage of IgA and secretory component in carcinomas of the female breast. *Virchows Archiv. Section A. Pathological Anatomy and Histology,* 393, 315-320.

SZIRMAI, J.A. & BALAZS, E.A. (1958). Metachromasia and the quantitative determination of dyebinding. *Acta Histochemica Supplementband*, 1, 56-79.

TABÁR, L. & DEAN, P.N. (1982). Mammographic parenchymal patterns. Risk indicator for breast cancer? *Journal of the American Medical Association*, 247, 185-189.

TANAKA, Y. & OOTA, K. (1970). A stereomicroscopic study of the mastopathic human breast. I. Three dimensional structures of abnormal duct evolution and their histologic entity. *Virchows Archiv. Section A. Pathological Anatomy and Histology*, 349, 195-214.

TANNER, J.M. (1962). Growth at Adolescence. 2nd Ed. p.36. Blackwell Scientific Publications, Oxford.

TASHIMA, C.K. (1974). Trauma and cancer. *Lancet* 2, 590-591.

TAVASSOLI, F.A. & NORRIS, H.J. (1980). Secretory carcinoma of the breast. *Cancer*, 45, 2404-2413.

TAYLOR, H.C. Jr. (1936). The relation of chronic mastitis to certain hormones of the ovary and pituitary and the coincident gynecological lesions. *Surgery, Gynecology and Obstetrics*, 62, 129-148.

TAYLOR, H.C. & WALTMAN, C.A. (1940). Hyperplasias of the mammary glands in the human being and in the mouse. Morphologic and etiologic contrasts. *Archives of Surgery*, 40, 733-821.

TELLEM, M., PRIVE, L. & MERANZE, D.R. (1962). Four quadrant study of breasts removed for carcinoma. *Cancer*, 15, 10-17.

THEELE, C. & BÄSSLER, R. (1981). Über grössenordnung, formen und varianten der drüsenlappchen der mamma. *Der Pathologe,* 2, 208-219.

THOMPSON, D.W. (1942). On Growth and Form. 2nd Ed. University Press, Cambridge.

THORN, G.W., NELSON, K.R. & THORN, D.W. (1938). A study of the mechanism of edema associated with menstruation. *Endocrinology,* 22, 155-163.

THREATT, B., NORBECK, J.M., ULLMAN, N.S., KUMMER, R. & ROSELLE, P. (1980). Association between mammographic parenchymal pattern classification and incidence of breast cancer. *Cancer,* 45, 2550-2556.

TOMPSETT, D.H. (1970). Anatomical Techniques. 2nd Ed. E.& S. Livingstone, Edinburgh, London, New York.

TRUSCOTT, B.M. (1947). Carcinoma of the breast. *British Journal of Cancer,* 1, 129-145.

TURNER, C.W. (1933). The normal and experimental development of the mammary gland of the monkey *(macacus rhesus). Anatomical Record,* 55, 80.

UNDERWOOD, J.C.E. (1972). A morphometric analysis of human breast carcinom. *British Journal of Cancer,* 26, 234-237.

URBAN, J.A. (1967). Bilaterality of cancer of the breast. Biopsy of the opposite breast. *Cancer,* 20, 1867-1870.

VALETTE, A., SERADOUR,B. & BOYER,J. (1975). Plasma testosterone levels during the menstrual cycle. *Journal of Clinical Endocrinology and Metabolism,* 40, 160-161.

VANDE WIELE, R.L., BOGUMIL, J., DYRENFURTH, I., FERIN, M., JEWELEWICZ, R., WARREN, M., RIZKALLAH, T. & MIKHAIL, G. (1970). Mechanisms regulating the menstrual cycle in women. *Recent Progress in Hormone Research,* 26, 63-103.

VAN HEUVERSWYN, J., FOLLEY, S.J. & GARDNER, W.U. (1939). Mammary growth in male mice receiving androgens, estrogens and desoxycorticosterone acetate. *Proceedings of the Society for Experimental Biology and Medicine,* 41, 389-392.

VAN WAGENEN, G. & FOLLEY, S.J. (1939). The effect of androgens on the mammary gland of the female rhesus monkey. *Journal of Endocrinology,* 1, 367-372.

VOGEL, P.M., GEORGIADE, N.G., FETTER, B.F., VOGEL, F.S. & McGARTY, K.S. (1981). The correlation of histologic changes in the human breast with the menstrual cycle. *American Journal of Pathology,* 104, 23-24.

VORHERR, H. (1974). The Breast: Morphology, Physiology and Lactation. Academic Press, New York.

de WAARD, F. (1975). Breast cancer incidence and nutritional status with particular reference to body weight and height. *Cancer Research,* 35, 3351-3356.

de WAARD, F. (1979). Premenopausal and postmenopausal breast cancer: one disease or two? *Journal of the National Cancer Institute,* 63, 549-552.

de WAARD, F. & BAANDERS-VAN HALEWIJN, E.A. (1974). A prospective study in general practice on breast-cancer risk in postmenopausal women. *International Journal of Cancer,* 14, 153-160.

de WAARD, F., CORNELIS, J.P., AOKI, K. & YOSHIDA, M. (1977). Breast cancer incidence according to weight and height in two cities of the Netherlands and in Alchi Prefecture, Japan. *Cancer,* 40, 1269-1275.

WALKER, F. (1968). The effects of hormones on inter-cellular ground substance. *Hospital Medicine,* 2, 589-593.

WARREN, S.L. (1930). A roentgenologic study of the breast. *American Journal of Roentgenology*, 24, 113-124.

WIEBEL, E.R. & ELIAS, H. (1967). Quantitative Methods in Morphology. Springer-Verlag. Berlin, Heidelberg, New York.

WELLINGS, S.R. (1980). Development of human breast cancer. *Advances in Cancer Research*, 31, 287-314.

WELLINGS, S.R. & JENSEN, H.M. (1973). On the origin and progression of ductal carcinoma in the human breast. *Journal of the National Cancer Institute*, 50 1111-1118

WELLINGS, S.R., JENSEN, H.M. & MARCUM, R.G. (1975). An atlas of sub-gross pathology of the human breast with special reference to possible precancerous lesions. *Journal of the National Cancer Institute*, 55, 231-273.

WELLINGS, S.R., JENSEN, H.M. & DEVAULT, M.R. (1976). Persistant and atypical lobules in the human breast may be precancerous. *Experientia*, 32, 1463-1465.

WELLINGS, S.R. & WOLFE, J.N. (1978). Correlative studies of the histological and radiographic appearance of the breast parenchyma. *Radiology*, 299-306.

WHITE, A., HANDLER, P., SMITH, E.L., HILL, R.L. & LEHMAN, I.R. (1978). Principles of Biochemistry. 6th Ed. p. 1134. McGraw-Hill, Kogakusha Ltd., Tokyo.

WHITLEY-BRANWOOD, A. (1963). The fibroblast. *International Review of Connective Tissue Research*, 1, 1-28.

WIGLE, D.T. (1977). Breast cancer and fertility trends in Canada. *American Journal of Epidemiology*, 105, 428-438.

WILKINSON, E., CLOPTON, C., GORDONSON, J., GREEN, R., HILL, A. & PIKE, M.C. (1977). Mammographic parenchymal patterns and the risk of breast cancer. *Journal of the National Cancer Institute,* 59, 1397-1400.

WILLIAMS, R.F. (1975). The Shoot Apex and Leaf Growth. p. 42. Cambridge University Press.

WILSON, J.L. (1981). Breast. In: *Current Surgical Diagnosis and Treatment, 5th Ed, edited by Dunphy, J.E., Way, L.W.* pp. 250-266. Lang Medical Publishers, Los Atlos, California.

WILSON, M.C. (1976). Observations on the normal breast. M.D. Thesis, University of Manchester.

WILSON, R.E. (1981). The breast. In: *Davis-Christopher Textbook of Surgery, 12th Ed., edited by Sabiston D.C.* pp. 622-665. W.B. Saunders Co. Ltd., Philadelphia.

WINSBERG, F., ELKIN, M., MACEY, J., BORDAZ, V & WEYMOUTH,W. (1967). Detection of radiographic abnormalities in mammograms by means of optical scanning and computer analysis. *Radiology,* 89, 211-215.

WISLOCKI., G.B., BUNTING, H., & DEMPSEY, E.W. (1947). Metachromasia in mammalian tissues and its relationship to mucopolysaccharides. *American Journal of Anatomy,* 81, 1-39.

WISLOCKI, G.B., BUNTING, H.& DEMPSEY, E.W. (1950). The chemical histology of the human uterine cervix with supplementary notes on the endometrium. In: *Menstruation and Its Disorders, edited by Engle, E.T.* pp. 23-50. Charles C. Thomas, Springfield, Illinois.

WOLFE, J.N. (1976a). Risk for breast cancer development determined by mammographic parenchymal pattern. *Cancer,* 37, 2486-2492.

WOLFE, J.N. (1976b). Breast patterns as an index of risk for developing breast cancer. *American Journal of Roentgenology,* 126, 1130-1139.

WOLFE, J.N. (1976c). Breast parenchymal patterns and their changes with age. *Radiology,* 121, 545-552.

WOLFE, J.N. (1979). Breast parenchymal patterns: prevalent and incident carcinomas. *Radiology,* 131, 267-268.

WOLFE, J.N. & WILKIE, R.C. (1978). Breast classification and observer error. *Radiology,* 27, 343-344.

WOLFE, J.N., ALBERT, S., BELLE,S. & SALANE, M. (1982). Breast parenchymal patterns: analysis of 332 incident breast carcinomas. *American Journal of Roentgenology,* 138, 113-118.

WYNDER, E., BROSS, I. & HIRAYAMA, T. (1960). A study of the epidemiology of cancer of the breast. *Cancer,* 13, 559-601.

YAMADA, M. & YOSHIDA, S. (1972). Graphic stereo reconstruction of serial sections. *Journal of Microscopy,* 95, 249-256.

ZACHARIAE, F. (1958). Autoradiographic (^{35}S) and histochemical studies of sulphomucopolysaccharides in the rabbit uterus, oviducts and vagina. Variations under hormonal influence. *Acta Endocrinologica,* 29, 118-134.

ZEPPA, R. (1969). Vascular response of the breast to estrogen. *Journal of Clinical Endocrinology and Metabolism,* 29, 695-700.

ZEPPA, R. & WOMACK, N.A. (1962). The role of histamine release in chronic cystic mastitis. *Surgery,* 52, 195-202.

ZIMMERMANN, M.H. & BROWN, C.L. (1971). Trees. Structure and Function. Springer-Verlag, Berlin, Heidelberg, New York.

ZUCKERMAN, S., VAN WAGENEN, G. & GARDINER, R.H. (1938). The sexual skin of the rhesus monkey. *Proceedings of the Zoological Society of London. Series A,* 108, 385-402.

APPENDIX A

METHODOLOGY

I. ORIENTATION OF WHOLE BREASTS

Orientation of specimens is an important feature in any project involving morphology or quantitation. The reference point for orientation purposes in the breast is generally the nipple. In this study, the nipple was taken as representing the central point of the specimen. Perpendicular axes were drawn through the nipple to represent 12, 3, 6 and 9 o'clock positions on the mammary fat pad. The breast tissue could then be divided into quadrants:

upper outer quadrant (UOQ)

upper inner quadrant (UIQ)

lower outer quadrant (LOQ)

lower inner quadrant (LIQ)

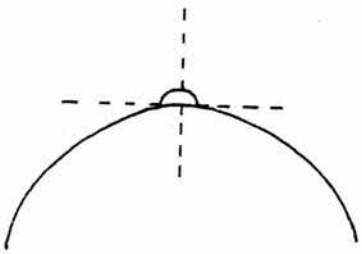
Considerable care was taken to ensure that the specimen did not lose its orientation at the time of surgical removal. Surgeons were requested to orientate the specimen by marking the "o'clock" positions and the UOQ. Good cooperation was obtained and all the specimens were marked at the points described either with sutures or with Indian ink. Autopsy specimens were also marked accordingly by those responsible for removal from the body. From the time of reception of a specimen in the Pathology Department, orientation was carefully maintained during processing as described in chapter 2.

Unless sophisticated techniques are used, it is impossible to estimate accurately to what extent a breast specimen is altered from its *in vivo* orientation on removal from the body. The specimens in this study were fixed while pinned to cork boards and therefore in a flat position. Such an orientation is comparable to one of the *in*

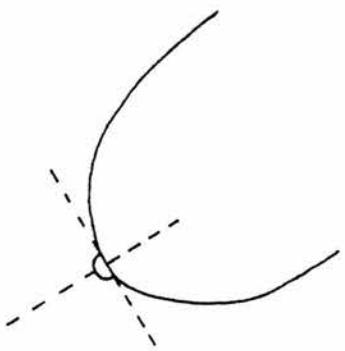
vivo positions a patient may assume during a medical breast examination, i.e. the patient supine with arm raised, the hand underneath the head. A similar positioning of the patient can be adopted for surgical mastectomy. It can be argued that a flat orientation of the breast is not comparable to the position of the breast on the chest wall *in vivo* with the patient in a sitting position.

The difference between a supine and a sitting position is illustrated below:

a) supine



b) sitting



It can be seen from the diagram that, in terms of quadrant definition, the two positions are not dissimilar. The perpendicular through the nipple divides the quadrants into much the same proportions in either situation and the nipple is indeed a good reference point.

The difference between the two positions is not in terms of quadrant size and definition, but rather in terms of distribution of tissue laterally and in depth. In the supine position (following surgical mastectomy), the nipple slumps somewhat on removal from the body; the breast becomes shallower and more tissue is displaced laterally than would be the case *in vivo*. Specimens removed at autopsy do not present this problem, as the tissue once dissected from the body remains firm and holds its shape well.

A technique to improve the accuracy of orientating the breast was considered. A mould in dental alginate (Zelgan, A.D. International Ltd., Weybridge, Surrey) of the diseased breast from four patients was obtained prior to mastectomy. The impression was taken with patients sitting upright. The nipple, UOQ and the "o'clock" positions were marked on the mould which was then stored in damp paper towels to avoid distortion. During mastectomy, the surgeon orientated the breast as to the nipple, UOQ and "o'clock" positions. Following mastectomy, the alginate mould was punched with holes using a large gauge cork borer and the breast placed inside it with the orientation marks for both corresponding. The breast in its mould was inserted in "Carson's formalin fixative" (Carson *et al.*, 1973), with a perforated metal plate placed on top to hold the specimen beneath the formaldehyde. The perforations through the mould and metal plate allowed penetration of the fixative into the specimen. Processing then proceeded as usual through the fixation, embedding, slicing, staining and clearing stages as described in chapter 2.

Two limitations of the technique emerged. First and most important was the trauma to the patient in having to undergo such a procedure. Secondly, it was difficult or impossible to take moulds from patients with pendulous breasts in a sitting position. On the basis of these two problems, it was decided not to pursue the technique; it was unlikely that all the patients in a proposed study would successfully provide moulds of their breasts and, further, it was considered not psychologically fair to the patients to subject them to the procedure, even although they were volunteers.

The four moulds successfully obtained were from women below 40 years of age having non-pendulous breasts. The technique confirmed the observation that the breast slumps to a varying extent following surgical mastectomy. It was discovered that the position of the nipple when the breast was flat was not markedly different from the nipple position in the mould, and that it was indeed the lateral displacement of the tissue and the depth of the breast which were altered.

It was decided for the purposes of this study that the techniques of orientation and fixation employed represented as adequately as practicable the *in vivo* condition and also were easily reproducible over a series of specimens.

II. FIXATION AND SLICING OF WHOLE BREASTS

The sooner after mastectomy that the breast is sliced, the greater is the surface area available for fixation. Penetration of fixative to the central parts of a specimen as rapidly as possible is desirable to obtain optimum fixation and good histology. Several régimes for fixation and techniques for slicing the breasts were attempted.

a) Two fresh mastectomy specimens were wrapped in tinfoil and placed in the freezing compartment of a refrigerator at -10^0C for 4 hours. The specimens were then sliced sagitally using a domestic meat slicer (Salter Housewares Ltd., Tonbridge, Kent). It was found that the specimens were difficult to cut and defrosted quickly. This made handling awkward and led to slices of uneven thickness. The slices became very friable on defrosting and it was not possible to achieve slices less than 5 mm thick which would withstand processing. Most importantly, histology obtained from the specimens was very poor due to freezing artefacts.

b) Two fresh mastectomy specimens were placed in the freezing compartment of a refrigerator at -10^0C for 1 hour and the top and bottom surfaces of the specimens then cooled until firm with CO_2 snow. The breasts were sliced on the meat slicer inside a cold room (5^0C) to prevent thawing until slicing was completed.

The results were as unsatisfactory as the first experiment. Histology was still poor, the slices very friable and no improvement was made on the 5 mm thickness. An additional disadvantage, that

fat does not freeze well in a short period of time, became apparent. It was concluded that tissue would have to be fixed prior to slicing.

c) Two fresh mastectomy specimens were fixed for 24 hours in Carson's fixative and then sliced with the meat slicer at room temperature. Grease from the mammary fat pad made handling and control very difficult. Slices of 4 mm thickness were achieved. A third mastectomy specimen fixed for 24 hours in Carson's fixative and surface cooled with CO_2 snow proved easier to handle on slicing, but with no improvement on the 4 mm thickness.

d) A fresh mastectomy specimen was fixed for 24 hours in Carson's fixative, quick frozen in liquid N_2 and then sliced. The specimen was so hard that it did not cut easily and the slices obtained broke up on defrosting. It was hoped that this rapid freeze technique would not affect histology, but the method proved impracticable and was not pursued.

e) It was found that breasts which had been fixed in Carson's fixative for 5 days cut very much better and did not require to be frozen. Longer fixation increased the firmness of the specimen, both on the surface and in the central areas which after 5 days had become fixed. An improvement was not made on the 4 mm thickness of the slices obtained, but handling was very much facilitated.

A 250 mm gravity commercial slicer (Excel Equipment Ltd., Hale, Chester) was obtained. This represented a considerable advance on the domestic meat slicer, but with the breasts fixed for 5 days, no improvement was made on the 4 mm thickness of the slices.

It was concluded that, in order to obtain 2 mm thick slices, the breasts would have to be embedded in gelatin prior to slicing. It was also hoped that, with the aid of a gelatin embedding technique, the fixation time before slicing could be reduced to 72 hours, so that after slicing formalin could reach central areas of the breast as quickly as possible. It was decided to avoid damaging the breast by piercing the tissue in any way to promote fixative reaching internal structures prior to slicing.

III. GELATIN EMBEDDING

The correct concentration of gelatin for embedding the breasts was estimated at 12.5% as a result of a series of experiments.

Experiment 1: Optimum concentration of gelatin

Gelatin (BDH Chemicals Ltd., Poole, England) solutions of 25%, 20%, 12.5%, 10% and 5% were made up in warm water, stored in flat bottom, 7.5 x 2.5 cm, glass tubes at 60^0C and stirred occasionally until dissolved. The solutions were then cooled in a 4^0C refrigerator until set.

a) A needle attached to a weight (total weight 14.5 g) was dropped onto each tube from 2.5 cm height to test the firmness of the gelatin, both at controlled room temperature (20^0C) and 4^0C. There was 50 ml solution in each tube.

The results are given below.

Gelatin %	Penetration at 4^0C (in mm)	Penetration at RT (in mm)
25	5	7
20	10	11
12.5	13	24
10	20	24
5	32	24

b) All the tubes were immersed in hot running tap water for 15 min and the results were as follows.

Gelatin %	Residue undissolved gelatin after 15 min %
25	20
20	5
12.5	0
10	0
5	0

Experiment a) indicated that the gelatin needed to be at 4^0C to maintain its firmness during the slicing procedure. Experiment b) showed that 12.5% gelatin was the highest concentration to dissolve with hot running tap water.

Experiment 2: Reversibility of gelatin/effect of formalin

From formalin fixed tissue, 10 similar pieces were selected; 5 were washed in running tap water for 4 hours and the remainder were left in fixative during this period. All 10 were then embedded in gelatin, one piece from each group in a different concentration. Five tubes contained gelatin only at 25%, 20%, 12.5%, 10% and 5% concentrations and 5 tubes contained 0.4% formalin mixed with gelatin at the stated concentrations. When set, all the tubes were placed in a water bath at 70^0C for 30 minutes. All the liquid gelatin was then removed and the tubes examined for gelatin which had remained undissolved. The results are tabulated below.

% Gelatin	Washed tissue	Unwashed tissue	No tissue	Formalin
25	liquified	solid 5 mm around tissue	liquified	solid
20	liquified	solid 5 mm around tissue	liquified	solid
12.5	liquified	liquified	liquified	solid
10	liquified	liquified	liquified	solid
5	liquified	liquified	liquified	liquified

The experiment showed that unwashed tissue and even a very weak solution of 0.4% formalin can render gelatin an irreversible gel. A 12.5% gelatin solution was the highest concentration at which gelatin became liquified on washing.

Experiment 3: Reversibility of gelatin/effect of time

Four pieces of formalin fixed tissue were washed for 2 hours in cold running tap water and each piece placed in a tube of 12.5% gelatin. After setting at 20^{0}C, a tube was placed under hot running tap water at intervals of 4, 24, 48 hours and 7 days to establish whether the gelatin was still reversible. The results were as follows.

	4 h	24 h	48 h	7 days
Gelatin:	liquified	Irreversible for 3-4 mm around the tissue	Irreversible for 5 mm around the tissue	Irreversible for 1 cm around the tissue

The results indicated that the gelatin should be washed out as soon as possible after embedding and slicing.

Experiment 4: Method for gelatin embedding

A whole fixed breast was washed for 2 hours in warm running tap water, then placed in a tub of cold water in an oven overnight at 37^0C. The breast was embedded in 12.5% gelatin and placed in a refrigerator at 4^0C until set. Half the breast was sliced coronally and half sliced sagittally on the commercial slicer at 2 mm thickness. Slicing was successful. The gelatin was washed out with hot running tap water within 15 minutes and all the gelatin successfully dissolved.

This was established as the method of choice and, on sectioning and processing blocks of tissue, it was found that the short time at 4^0C had not adversely affected the histology.

IV. THICKNESS OF BREAST SLICES

The variation in thickness of slices of breast tissue was assessed using the following equipment and method:

Metric dial gauge

Two 32 oz glass plates each 15 cm^2

Sheet acetate film, 14 cm^2

A slice of breast tissue was placed on one of the glass plates. Using a fine tip felt pen with permanent ink, small crosses 2 cm apart were marked on the sheet of acetate film. The film was then placed over the slice of tissue in such a manner that as many crosses as possible were positioned on top of the slice. The second glass plate was placed on top of the acetate film and both glass plates sellotaped together to hold the slice and film firmly in position.

The thickness of the slice on any 10 of the crosses lying on top of it was measured using a metric dial gauge (Mercer Ltd., St Albans, Herts.), which had previously been centred on the glass plates and acetate film alone. The foot of the dial gauge measured 19 mm and it was found that 10 crosses 2 cm apart adequately represented an average slice. Readings were taken as soon as stability was achieved so that effects of tissue compression did not influence the results.

Five breast slices were selected at random and measured:

1) after slicing and still embedded in gelatin

2) after the gelatin had been washed out

3) after staining and clearing. The cleared slices were heat sealed in transparent bags (Kapak Corporation, Bloomington, USA) prior to measurement.

The results, which are shown below, are expressed in mm and with standard errors.

Slice	With gelatin	Without gelatin	Cleared
1	2.08 ± 0.17	1.81 ± 0.10	2.02 ± 0.10
2	2.03 ± 0.12	2.01 ± 0.06	2.16 ± 0.08
3	2.34 ± 0.15	2.06 ± 0.09	2.11 ± 0.15
4	2.27 ± 0.10	1.95 ± 0.06	2.05 ± 0.05
5	2.48 ± 0.12	2.08 ± 0.13	2.35 ± 0.07
For all the slices	2.23 ± 0.06	1.98 ± 0.04	2.14 ± 0.04

These results supported the impression that the thickness of the slices varied according to the density of the tissue which was being cut. Areas of fat sliced marginally thinner than areas of fibrous tissue. Also, the edges of slices were thicker than the central areas.

The figures recorded for the slices still embedded in gelatin have the largest measurements. Whilst obtaining these figures, note was taken of which readings were taken at the edge of slices and which from central areas. It was confirmed that the thicknesses recorded at the edges of the slices were greater than readings recorded in more central portions. On washing out the gelatin, the overall average thickness of the slices was reduced and approximated very closely to the desired thickness of 2 mm. The slice edges were not found to be consistently thicker than central zones. It was concluded that gelatin was responsible for this discrepancy in thickness during slicing. Although a vacuum gelatin embedding technique was not used, gelatin did infiltrate between fat lobules

on the surface of the breast and, on slicing, it occasionally occurred that gelatin overlapped the edge of the breast tissue, giving a false impression that the edge of the slice was thicker than the central areas. After clearing the slices, a little more variation in thickness was found, but the results were still quite acceptable and still approximated well to 2 mm. Distortion of the tissue during processing was not found to be a factor for concern with this technique.

Holding the specimen in position whilst feeding it into the slicing machine does place some pressure on the tissue during slicing. The resultant tissue compression could account for some variation in the thickness of slices and explain why fatty areas may slice marginally thinner than areas of fibrous tissue. The blade of the slicer is also to some extent flexible and the tissue must be fed slowly into the machine to avoid uneven slices. To prevent "chattering" of the tissue during slicing, the blade must be sharpened regularly.

V. STAINING OF TISSUE FOR SUBGROSS EXAMINATION

Other workers who have adopted slicing techniques (Ingleby & Holly, 1939; Gough & Wentworth, 1949; Egan, Ellis & Waldo Powell, 1969; Marcum & Wellings, 1969; Egan, 1970; Wellings *et al.*, 1975) have all used Harris' haematoxylin to stain their tissue sections. Using the technique of Wellings *et al.*,(1975) as a basis for experiment, a series of staining trials was performed using various dilutions of Harris' haematoxylin (Culling, 1963) and various staining times. It was concluded that a 50% dilution in tap water produced the best staining result with good definition of parenchymal structures and relatively unstained background fibrous stroma. Further extensive experimentation was performed to ascertain the correct strength of acid ethanol for differentiation as well as the optimum schedule for ethanol dehydration of the tissue slices.

Unfortunately, in the course of time, a serious disadvantage in the use of Harris' haematoxylin became apparent. After only a short period in storage, the stain leached out of the epithelial structures to discolour the connective tissue, rendering the slice a uniform dark red colour. As several weeks were anticipated as the minimum period required for the examination of the tissue, this feature was obviously undesirable. It was decided that a different haematoxylin would be tried and staining experiments were repeated using varying dilutions of Delafield's haematoxylin (Culling, 1963). A 50% dilution of this stain for 45 minutes, followed by differentiation in 2% acid ethanol and washing for 2 - 4 hours in running cold tap water, was found to be most successful, and produced a better

quality of staining which did not leach into surrounding tissues on prolonged storage.

By staining and clearing tissue of varying thickness from 1 mm to 4 mm, it was determined that the optimum results were obtained with tissue at a thickness of 2 mm. At 1 mm thickness, the tissue was rather too thin to withstand the processing procedures without damage and some slices tended to break up with handling. At 3 and 4 mm it was not possible to see throughout the depth of a slice with a stereomicroscope (M8; Wild-Heerbrugg; Leitz Ltd., Luton, England). Also, the stain was not effective in penetrating the thickness of the tissue to produce an even staining result. At 2 mm thickness, all areas of a slice could be viewed with the stereomicroscope without difficulty; the tissue withstood processing procedures well and staining was at an acceptable level of evenness throughout.

When considering quantitation procedures, the extent to which all areas of a slice were visible was important. The 2 mm thickness was ideal as it allowed all the parenchyma in a slice of tissue to be examined and assessed.

Although standard preparations at varying concentrations of Harris' and Delafield's haematoxylin gave the best results, it should be noted that other staining methods were also tried.

1. Methyl green, 0.1%
   Stain 30 min.
   Wash in cold tap water 5 h.

2. Methyl green, 0.5%, aqueous.
   Stain 30 min.
   Wash in cold tap water 5 h.

3. Solochrome cyanine, 0.2%.
   Stain 15 min.
   Wash in cold tap water 5 h.

4. Solochrome cyanine, 0.2%, in 4% iron alum + 0.5 ml conc. H_2SO_4.
   Stain 2 h.
   Wash in cold tap water 30 min.

5. Carmine 2% in distilled water + 2 ml aniline oil.
   Stain 5 min.
   Wash in cold tap water 5 h.

6. Basic fuchsin, 0.05%.
   Stain 20 min.
   Wash in cold tap water 5 h.

7. Light green, 0.05%.
   Stain 10 min.
   Wash in cold tap water 5 h.

8. Sudan IV, saturated in absolute ethanol.
   30 ml diluted in 70% ethanol for use.
   Filtered.
   Slices pre-rinsed in 70% ethanol.
   Stain 1 h.
   Post rinse in 70% ethanol 10 min.
   Wash in cold tap water for 2 h.
   Counterstain with haematoxylin.

9. Alcian blue, 0.05%, in 0.5% acetic acid.
   Stain 15 min.
   Wash in cold tap water 5 h.

10. Alcian blue, 1%, in 2% acetic acid.
    Stain 30 min.
    Wash in cold tap water 5 h.

11. Toluidine blue, 1%, aqueous.
    Stain 30-45 min.
    Wash in cold tap water 30 min.

12. Toluidine blue, 1%, in 0.1% borax.
    Stain 10 min.
    Wash in cold tap water 30 min.

13. Anthracene blue, 0.5%, in 5% aluminium sulphate (boiled).
    Dilute 1:30.
    Stain overnight.
    Rinse 5% aluminium sulphate.
    Wash in cold tap water 30 min.
    Differentiate 1% aluminium chloride in 90% ethanol 30 min.
    Wash in cold tap water 14 h.

14. Harris' haematoxylin 100% standard preparation.
    Stain 1 h.
    1% acid ethanol differentiation.
    Wash in cold tap water 4 h.
    Counterstain with 0.5% eosin.

The following dehydration and clearing schedule was used in all cases:-

1. 95% ethanol 12 h.

2. 99.8% ethanol 12 h.

3. 99.8% ethanol 12 h.

4. 100% ethanol 12 h.

5. Clear in methyl salicylate minimum 6 h.

VI. CLEARING AGENTS

The background connective tissue had to be cleared before the stained parenchyma could be clearly identified with the stereomicroscope. The following clearing agents were investigated to see which was most suitable:-

methyl salicylate

cedar wood oil

xylene

toluene

acetone

methyl benzoate

turpentine

Of these, only methyl salicylate and cedar wood oil cleared the 2 mm breast slices effectively and reliably. Cedar wood oil proved to be too expensive and therefore methyl salicylate became the clearing agent of choice.

VII. CANNULATION OF LACTIFEROUS DUCTS AND DUCT INJECTIONS

Each mastectomy specimen which underwent this procedure was collected direct from theatre as a fresh specimen. The breast was orientated with Indian ink by marking the "o'clock" positions and the UOQ, and was radiographed using an exposure of $2\frac{1}{2}$ minutes at 25 kvp (X-ray tube anode voltage). The specimen was then placed in a 4^0C refrigerator to cool, which caused the mammary fat pad to firm considerably in consistency. On removal from the refrigerator, the breast was placed on a cork board covered with a layer of damp cotton wool. A pad of cotton wool was used to support the breast tissue directly beneath the nipple, placing it in a prominent position relative to the surrounding tissue. The top half of the nipple was sectioned coronally and removed. Artery forceps were attached to the edge of the nipple remaining to put a stretch on the tissue. The breast was then prepared for cannulation of the ducts in the nipple to proceed.

Using the stereomicroscope, ducts were dilated one at a time with a blunt probe and cannulated with a 2 cm length of 0.75 mm diameter intravenous tubing (Portex Ltd., Hythe, Kent) to which a 25G3/8 hypodermic needle was attached. If necessary, the cannula was supported in position by a small quantity of damp cotton wool. When cannulation of all possible ducts was completed, a map was drawn, for orientation purposes, of the nipple with the cannula *in situ*. The position of the ducts which had been cannulated and the direction in which the ducts appeared to be travelling was noted. The ducts were numbered in a clockwise direction and these numbers

were also marked on the hub of the hypodermic needles *in situ*.

Each duct was injected manually with micropaque (Nicholas Labs Ltd., Slough, Bucks.) using a 10 ml syringe. The ducts were injected in a clockwise fashion according to the numbering on the hypodermic needles. After each injection the breast was radiographed. For breasts in which the demonstration of a single duct system was required, duct injection was ceased as soon as a single duct had been shown by radiography to have been successfully injected.

The pressure of injection used was up to the maximum which could be exerted manually. If a sudden release of fluid occurred at maximum force, injection was ceased immediately and a radiograph taken to ascertain whether a duct had been perforated and micropaque was entering the tissues. In all cases the quantity of fluid successfully injected was noted and this varied considerably in volume from 0.5 to 5 ml. Unsuccessful injections were also noted. If micropaque welled back out of a cannula or a cannula was forced out of a duct by injection pressure, excess micropaque was swabbed away with cotton wool immediately and the area cleaned with ethanol, in order to avoid tissue contamination affecting subsequent radiographs. Occasionally micropaque was seen spilling at the surface of the mammary fat pad during injection. This was caused by a duct at the surface having been severed on the removal of the breast at mastectomy. Such a leak was packed immediately with cotton wool and any excess micropaque cleaned from the tissue. Spillage of micropaque from ducts opening onto the base of the breast was

absorbed by the cotton wool on top of the cork board.

After each cannulation the breast was radiographed. On completion of the injections and radiographs, the breast was placed in Carson's fixative and processed according to the schedule already described for slicing, staining and clearing whole breast specimens in chapter 2. After 24 hours fixation it was still possible to cannulate and inject ducts if the radiographs indicated that such a procedure might improve filling of a duct already injected.

Injection Materials

A selection of injection materials was considered:-

1. Lipiodol

Lipiodol (May & Baker Ltd., Dagenham, Essex) is an iodised oil fluid injection material. Although its viscosity does not preclude successful injection, lipiodol is not an easy fluid to control. It poured out of any injected ducts which were sectioned during the slicing procedure and was colourless against breast tissue. This created a difficulty in adequately clearing spillage which appeared on and spoiled radiographs. Protecting the specimen with clear polythene film to control any spillage was not successful.

Lipiodol does not inhibit the setting of gelatin. A 5% gelatin solution was mixed with lipiodol and stored at 60^{0}C. On injection it was found that the solution was too viscous to be effective and the working time before the gelatin set was short. The little that was successfully injected tended to crumble out of ducts which were

sectioned during slicing. The gel, although rendered irreversible by exposure to formalin vapour, was not robust and did not withstand well the staining and clearing procedures.

2. Acrylic

The object in attempting to inject acrylic into ducts was twofold:-

a) To obtain a non viscous, radio-opaque and durable injection material which would withstand the slicing, staining and clearing procedures.

b) To ascertain whether an acrylic skeleton of ducts could be produced following injection of coloured acrylics and subsequent acid digestion of surrounding breast tissue.

The acrylic was made up by adding 3 ml methyl methacrylate (ICI, Pharmaceuticals Division, Macclesfield, Cheshire) containing 0.2% dimethyl para-toluidine to 3 ml methyl methacrylate containing 0.1% hydroquinone in which 0.3 g of benzoyl peroxide crystals (BDH Ltd., Poole, England) were dissolved. The working time of the solution was 30 minutes ± 5 minutes.

The acrylic was successfully injected, but in the small quantities within the ducts it was not radio-opaque. The addition of 1 ml of lipiodol to the mixture rendered the acrylic radio-opaque and did not inhibit the set. However, on slicing the specimen, the set acrylic was so brittle it tended to snap and fall out of the ducts. The dampness of the breast tissue may have contributed to some poor filling of the ducts seen on slice radiographs.

Inclusion of red or blue dye in the injection material was not successful. Added in sufficient quantities to produce a positive colour, the dyes inhibited or prevented setting. Added in sufficient quantities to permit setting, the resultant colour was indistinguishable from that of the breast tissue.

Given the knowledge that an acrylic solution would be very delicate and the experience that it was difficult to inject adjacent ducts, it was decided that it was unlikely that a coloured acrylic model of breast ducts could be achieved.

3. Micropaque

Micropaque (Nicholas Labs Ltd., Slough, Bucks.) is a white radio-opaque fluid commonly used for gastro-intestinal investigations as it possesses good mucosal adhesive properties. It consists of a ready to use dispersion containing 100% w/v barium sulphate with a 0.5-2.0 μm particle size.

Micropaque was successfully injected into cannulated ducts. It should not be diluted before use as this results in uneven filling of the ducts. Being a white material, its appearance at or away from the injection site is clearly visible and spillage is therefore easy to deal with. It sets to a firm material within the breast which is visible in ducts and which is retained well after slicing. Micropaque also withstands processing very well and is easily visible on cleared slices. Having so many advantages, micropaque was selected as the injection material of choice.

Injection Pressure

The pressure required for the injection of micropaque solution into breast ducts is difficult to measure. The quantity of fluid, if any, in the form of secretions present in the ducts at the time of injection is unknown. Ultimately a manual injection technique was found to be the most successful and the maximum pressure exerted during injection was estimated at 9.5 kg force using a grip dynamometer (Takeikiki Kogyo Ltd., Japan).

Although the simple manual technique was adopted, other methods were investigated:

1. Heparin pump

The Handley Intravenous Injector (Wheathampstead Sales Services Ltd., Letchworth, Herts.) is designed to drive the plunger of a 20 ml syringe a distance of 5 cm over a period of 12 hours. Use of a 60 ml syringe produces a rate of flow of 4 ml/hour. The apparatus was successful, but injection was very slow and only the rate of flow and not the pressure of injection was controlled.

2. Peristaltic pump

A Peristaltic pump (Pharmacia (Great Britain) Ltd., Hounslow, Middlesex) has a pulsating flow. It can be attached to a cannula *in situ* in the breast without difficulty but, like the heparin pump, it controls only the rate and not the pressure of injection.

3. Sphygmomanometer

This instrument proved very difficult to use with a cannula *in situ* and controls the pressure of injection rather than the rate of flow.

VIII. RADIOGRAPHIC TECHNIQUES

Throughout this project a Faxitron 43805 X-ray system (Hewlett-Packard Ltd., Wokingham, Berks.) and Industrex C radiographic film (Kodak Ltd., Glasgow, Scotland) have been used.

In all cases tissue was dried with paper towels before radiographic exposure, as the presence of water produces a distortion of the opacity on developed film.

Various trial exposures were performed on whole breasts and breast slices. It was found that the following exposures produced the most acceptable results:

a) For whole specimens up to 4½ cm in depth from the nipple to the base of the breast and radiographed flat: 2½ minutes at 20 kvp (X-ray tube anode voltage).

b) For specimens in excess of 4½ cm in depth and radiographed flat: 2½ minutes at 25 kvp.

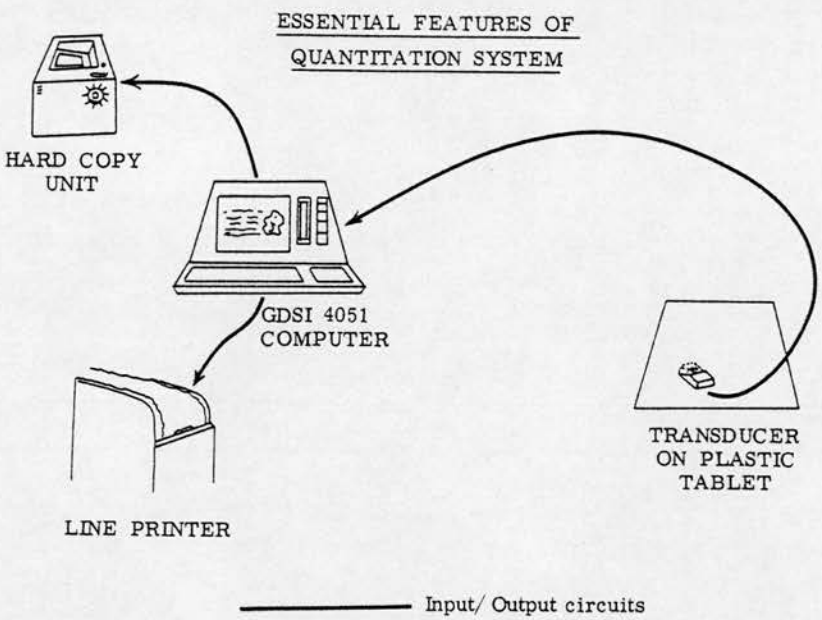
c) For breasts tilted at 45^0 from the horizontal: 3 minutes at 30 kvp.

d) Predominantly fibrous 2 mm thick breast slices: 45 seconds at 20 kvp.

e) Predominantly fatty 2 mm thick breast slices: 30 seconds at 20 kvp.

IX. GRAPHIC SYSTEMS 4051 SEMI-AUTOMATIC IMAGE ANALYSING SYSTEM

Quantitation of the non-fatty component (NFC) in the breast was achieved using a Graphic Systems 4051 semi-automatic Image Analysing System (Tektronix Ltd., Harpenden, Herts.) linked with an Anadex Alphanumeric lineprinter and Tektronix hard copy unit. The essential features of the quantitation system are illustrated below.



An intricate wire grid lying beneath a plastic tablet and used in conjunction with a hard-line transducer (Tektronix) allowed direct quantitation from tracings of breast tissue slices. The heat generated by back lighting radiographs placed on top of the tablet eliminated the direct measurement of NFC from radiographs as a technique. During quantitation, a stream of points (co-ordinates) passes from the plastic tablet and wire grid to the computer where mathematical analysis occurs. The position of the transducer on

the plastic tablet is represented on the video screen of the computer by a pinpoint of light derived from a small light bulb on the transducer itself. It is this pinpoint of light which outlines the profile of the NFC which is being traced and causes the generation of co-ordinates through an electromagnetic effect on the transducer in contact with the tablet. The video screen of the computer thus automatically displays an image of structures being quantified.

Data are produced in a permanent form as copy from the line-printer and also stored on a magnetic tape cartridge. If required, hard copies (a type of photocopy) may be made of the information displayed on the video screen of the computer. These give a permanent record, although not to scale, of the shape of an area quantified.

The programme used for the quantitation procedure is a modification of the general software package in the Qualitative Analysis System User Manual provided by Graphics Information Systems Ltd. (Blairgowrie, Scotland) who were responsible for installation of the system and who maintain it. The computer is programmed using the BASIC language (Beginners All-purpose Symbolic Instruction Code) which is a moderately simple computer language.

The system was easily calibrated prior to use as all measurements were to scale; and therefore calibration was 1, i.e. unity.

X. ERRORS INVOLVED IN THE MEASUREMENT OF THE TOTAL AREA AND THE AREA OF THE NON-FATTY COMPONENT (NFC) IN THE BREAST

1. Use of the GDSI 4051 Machine

A series of shapes both of known and unknown area were measured on the GDSI 4051 machine to establish those errors involved in the technique due to the operator and those arising from the machine itself. Only for repetition of irregular areas of approximately 1 cm^2 did an error exceed 5% and this figure decreased the greater the number of readings taken. Only for smaller areas in the order of 4 mm^2 did the error exceed 10%. The great majority of readings in the study were well within a 5% margin of error and were therefore acceptable.

2. Reproducibility of Non-Fatty Component (NFC) Measurements from Radiographs

Although measurements of the total area of slices were reproducible to within 2% (because of the large, regular shapes involved), the results for NFC were more variable and reached as high as 16.23% variation for smaller slices which contained little NFC.

However, although reproducibility of small slices was at times poor, over a series of slices the reproducibility was good. Half a breast, consisting of 19 slices, was traced from radiographs twice (on different days) and the areas of NFC measured. Variation between the two sets of measurements was for the upper quadrant, 2.31% and for the lower, 2.1%. Taking the results of the smallest 10

slices, variation was for the upper, 5.7% and the lower, 1.1%. For the rest of the slices, i.e. the largest, variation was for the upper, 0.34% and the lower, 2.75%.

It may be concluded that, whether the breast was small and contained few slices or vice versa, assessment of the area of NFC made from radiograph tracings was reliable.

XI. EMBEDDING CYCLE AND STAINING RÉGIME FOR BREAST BIOPSIES

All formalin and unstained tissues were processed automatically on a Histokinette (Reichert-Jung, Slough, Bucks.) using a 24 hour embedding cycle. The following 12 stages were used:-

1.	70% ethanol	3 hours	
2.	96% ethanol	3 hours	
3,4.	99% ethanol	2 hours	x 2
5,6.	absolute ethanol	2 hours	x 2
7,8.	absolute ethanol/ chloroform equal parts	2 hours	x 2
9,10.	chloroform	2 hours	x 2
11,12.	paraffin wax at 60^0C	30 min	

Tissues which had been subjected to staining and clearing procedures were placed in absolute ethanol for 24 hours; added to the Histokinette schedule at stage 6 and thereafter processed routinely to stage 12 as described.

Staining Régime for Biopsies

A total of 70 blocks represented the 43 cases in this study. For each block, 6 serial sections at 3 µm were prepared and mounted for staining and labelled 1 - 6 as appropriate.

a) Haematoxylin and Eosin (H & E)

For all 70 blocks, section 1 was stained in a routine manner with H & E.

b) Combined Alcian Blue/Periodic Acid-Schiff (AB/PAS) Method

For all 70 blocks, section 2 was stained with AB/PAS. Staining was performed in 4 batches of 14 sections along with 2 control sections from 2 diagnosed cases of breast fibroadenomata known to display strong AB/PAS staining. To provide an additional check of staining consistency, section 6 from 3 cases selected at random in each batch were used to overlap into the next batch. There was therefore a total of 19 sections in each batch.

Every block with its sections was coded in a simple manner so that the operator making histological assessments was unaware of which blocks had been included in which batch and, more importantly, had no knowledge of menstrual cycle status and other relevant data.

The same batches of alcian blue and basic fuchsin stains were used throughout all the experimentation in this project. The AB/PAS sections were stained during the course of one day using a single Schiff solution which was freshly prepared. Temperatures and timing were strictly controlled.

c) Diastase Digestion followed by Alcian Blue/Periodic Acid-Schiff (AB/PAS) Staining

Section 3 from 20 blocks selected for their high scores of secretions and/or intracellular granules underwent malt diastase digestion followed by AB/PAS staining. One section from each of

2 normal human liver biopsies derived from autopsy were included as positive controls.

d) Hyaluronidase Digestion followed by Toluidine Blue Staining

Section 3 from 10 blocks selected for their prominent staining of the intralobular stroma underwent testicular hyaluronidase digestion followed by toluidine blue staining at pH 2.0. One section from each of 2 blocks of umbilical cord were included as positive controls.

e) Toluidine Blue Staining

Section 3 from 20 blocks with positive scores for intracellular granules within the majority of cells was stained with toluidine blue in a routine manner.

f) Van Gieson's Method for Collagen

This stain was used on section 5 from 6 blocks displaying strong alcian blue staining and 6 blocks showing weak alcianophilia of the intralobular stroma.

g) Alcian Blue and the Periodic Acid-Schiff Reaction

These stains were used individually on 2 sections from both control blocks of fibroadenomatous tissue to provide a reference if required of the reactions of the 2 stains acting separately on breast tissue.

XII. HISTOCHEMICAL TECHNIQUES

a) Delafield's Alum Haematoxylin

This solution (Culling, 1963) is made up as follows:-

1. Ammonium alum 55 g and distilled water 600 ml.
2. Haematoxylin 6 g and ethanol 50 ml.
3. Glycerol 150 ml and ethanol 150 ml.

Prepare solutions 1, 2 and 3 separately. Mix 1 with 2 and leave overnight. Pass the mixture through filter paper and add solution 3. Store for 6 weeks in a light, warm place to ripen. The stain is described as having an "almost indefinite" shelf life.

b) Haematoxylin and Eosin (H & E)

This method was modified from Drury & Wallington (1967):-

1. De-wax sections and take to water.
2. Stain in an alum haematoxylin (Harris') for 5-15 minutes.
3. Wash well in running tap water for 2-3 minutes.
4. Decolourise in 0.5-1% HCl in 70% ethanol for a few seconds.
5. Rinse in running tap water.
6. Immerse in Scott's tap water substitute for approximately 30 seconds.
7. Rinse in running tap water.
8. Stain in 1% aqueous eosin 1-3 minutes.
9. Wash off surplus stain in tap water.
10. Dehydrate, clear and mount.

c) Alcian Blue (AB)

AB solution was made up fresh prior to each staining procedure. The solution was 1% AB (Raymond A. Lamb, London, England) in 3% acetic acid, pH 2.5. Slides were de-waxed, taken to tap water and stained by immersion at 20^0C (controlled room temperature) for 20 minutes.

d) The Periodic Acid-Schiff (PAS) Reaction

1. De-wax sections and take to water.
2. Treat with 1% aqueous periodic acid for 5 minutes.
3. Rinse in distilled water and treat with Schiff's reagent for 5 minutes.
4. Wash in running tap water for 2 minutes.
5. Stain nuclei with haematoxylin in the normal manner.
6. Dehydrate, clear and mount.

e) Combined Alcian Blue/Periodic Acid-Schiff (AB/PAS) Method

1. De-wax sections and take to water.
2. Stain with 1% AB (Raymond A. Lamb) in 3% acetic acid, pH 2.5, for 20 minutes at 20^0C.
3. Wash in tap water.
4. Oxidise with 1% aqueous periodic acid at 20^0C for 4 minutes.
5. Wash thoroughly with distilled water for 5 minutes.
6. Immerse in Schiff's reagent for 5 minutes at 20^0C.
7. Wash well in running tap water for 2 minutes.
8. Dehydrate, clear and mount.

f) Toluidine Blue

1. De-wax and take to water.
2. Rinse in 95% ethanol.
3. Stain in 1% toluidine blue for 10 minutes. (For pH 2.0 add Walpole's sodium acetate-hydrochloric acid buffer.
4. Blot dry.
5. Clear in xylene.
6. Mount.

g) Diastase Digestion with Alcian Blue/Periodic Acid-Schiff (AB/PAS) Method

The same batch of diastase (BDH Ltd., Poole, England) was used throughout. It contained α and β amylase with approximately 20% reducing sugars determined as glucose and had an activity of 4 EU/mg.

1. De-wax sections and take to distilled water.
2. Treat with a 1% solution of malt diastase in 0.02 M acetate buffer pH 5.5 overnight at 37^0C.
3. Wash well in running tap water.

Sections of human liver were processed in parallel to provide positive controls. Negative controls of breast fibroadenomata were treated for the same time and temperature in distilled water.

4. Perform the AB/PAS technique.
5. Dehydrate, clear and mount.

h) Hyaluronidase Digestion with Toluidine Blue

Bovine testicular hyaluronidase (Sigma Chemical Co., Poole, England) Type 1-S lyophilized powder was used. It had a sodium content of 0.10 wt % and was 450 NF units/mg solid. The same batch of hyaluronidase was used throughout the study.

1. De-wax sections and take to distilled water.
2. Treat with a 1% solution of bovine testicular hyaluronidase in 0.02 M acetate buffer pH 5.5 overnight at 37^0C.
3. Wash well.

Positive and negative controls of breast fibroadenomata were included. The negative control sections were treated for the same time and temperature with distilled water.

4. Perform toluidine blue staining at pH 2.0.
5. Dehydrate, clear and mount.

i) Van Gieson's Method for Collagen (Lillie, 1965)

1. Stain for 5 minutes in Weigert's iron haematoxylin.
2. Wash in tap water.
3. Stain for 5 minutes in picrofuchsin mixture. (Van Gieson's picrofuchsin mixture consists of 5 cc 1% acid fuchsin to 95 cc saturated aqueous picric acid solution.)
4. Dehydrate and differentiate with 2-3 changes each of 95% and 100% ethanol.
5. Clear with a mixture of 100% ethanol and xylene followed by 2-3 changes of pure xylene.
6. Mount.

XIII. FIXATION AND STAINING TRIALS FOR THE STUDY OF MUCOPOLYSACCHARIDES IN BREAST LOBULES

Fixation Time

A fresh mastectomy specimen was fixed for 24 hours in Carson's fixative. The breast was sliced and 12 blocks of tissue were removed from central areas of the specimen. None was sufficiently fixed to be processed for histology and they were therefore replaced in the Carson's fixative.

Six blocks of tissue were processed through paraffin wax after a further 24 hours fixation and the remainder after 48 hours. Haematoxylin and eosin (H & E) and alcian blue/periodic acid-Schiff (AB/PAS) stained sections were obtained. The sections were coded and assessed for staining quality without prior knowledge of the fixation times. The staining schedule for the AB/PAS is reproduced below and is that which is normally used in the routine biopsy laboratories in the University of Edinburgh Pathology Department.

1. De-wax sections and take to water.
2. Stain in 1% alcian blue in 3% acetic acid, pH 2.5, 20 minutes.
3. Wash in running tap water 2 minutes.
4. Oxidise in 1% periodic acid 3 minutes.
5. Wash in running tap water 1 minute.
6. Treat with Schiff's reagent in a closed container for 5 minutes at room temperature (approx. 20^0C).
7. Wash in running tap water 5 minutes.
8. Dehydrate, clear and mount.

There were no significant differences in staining between the AB/PAS sections. The PAS staining in all the sections was noted as being pale.

In a second experiment, a fresh autopsy specimen was sliced and 8 blocks of tissue were removed and placed in Carson's fixative for the following periods of time:

2 blocks for 18 h
2 blocks for 24 h
2 blocks for 42 h
2 blocks for 48 h

Blocks removed from the fixative prior to 48 hours were placed in 70% ethanol for storage and all the blocks were processed together. Routine paraffin sections stained with H & E and AB/PAS were obtained as in the previous experiment. The quality of all the sections was found to be similar and again revealed that the strength of the PAS staining was pale. It was concluded that, as all sections were of similar quality, the length of time spent in fixative for periods up to 72 hours duration was not a factor adversely affecting staining. It was decided to investigate whether the pH of a fixative could influence the results.

pH of Fixative

Formalin solutions were prepared at the following hydrogen ion concentrations:

pH 7.4 - Carson's fixative

pH 6.8 - 10% formalin in a phosphate buffer

pH 5.0 - this acidity of 10% phosphate buffered formalin was selected as it was discovered that the formalin used in the "routine" biopsy laboratories often descended from pH 6.8 to pH 5.0.

Tissue was taken from freshly killed rats used as controls in another research programme. The rats were ICI male strain, 250-280 g body weight. They were administered 0.5 ml saline IP and killed 12 hours later. Pieces of small intestine, colon and liver were dissected out and fixed for 24 hours in each of the fixatives above. The blocks of tissue were processed and routine paraffin sections obtained and stained with H & E and AB/PAS as before.

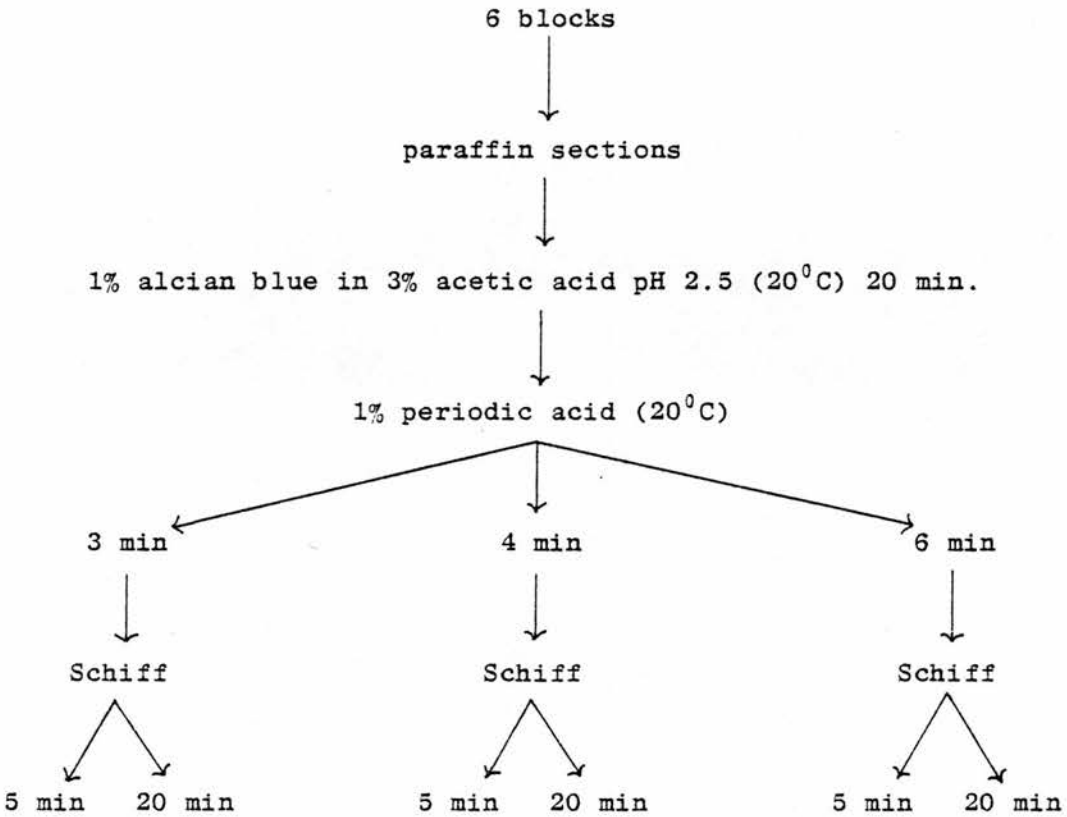
It was found that the pH of the fixative did not appear to be critical to the results. Again, the PAS staining was weak and the sections were all of a similar quality.

Carson's fixative at a controlled physiological pH 7.4 was selected as the fixative solution of choice. Alterations in pH of Carson's fixative if the solution was left to stand were recorded as reaching up to pH 8.7, but it was found that this fixative remained at a stable pH for longer periods of time than the other trial solutions and the pH was always checked prior to use.

Alcian Blue/Periodic Acid-Schiff Staining Schedule

Attention was focused on determining whether the poor quality of the PAS staining could be improved and on establishing a staining schedule which provided satisfactory and reproducible results.

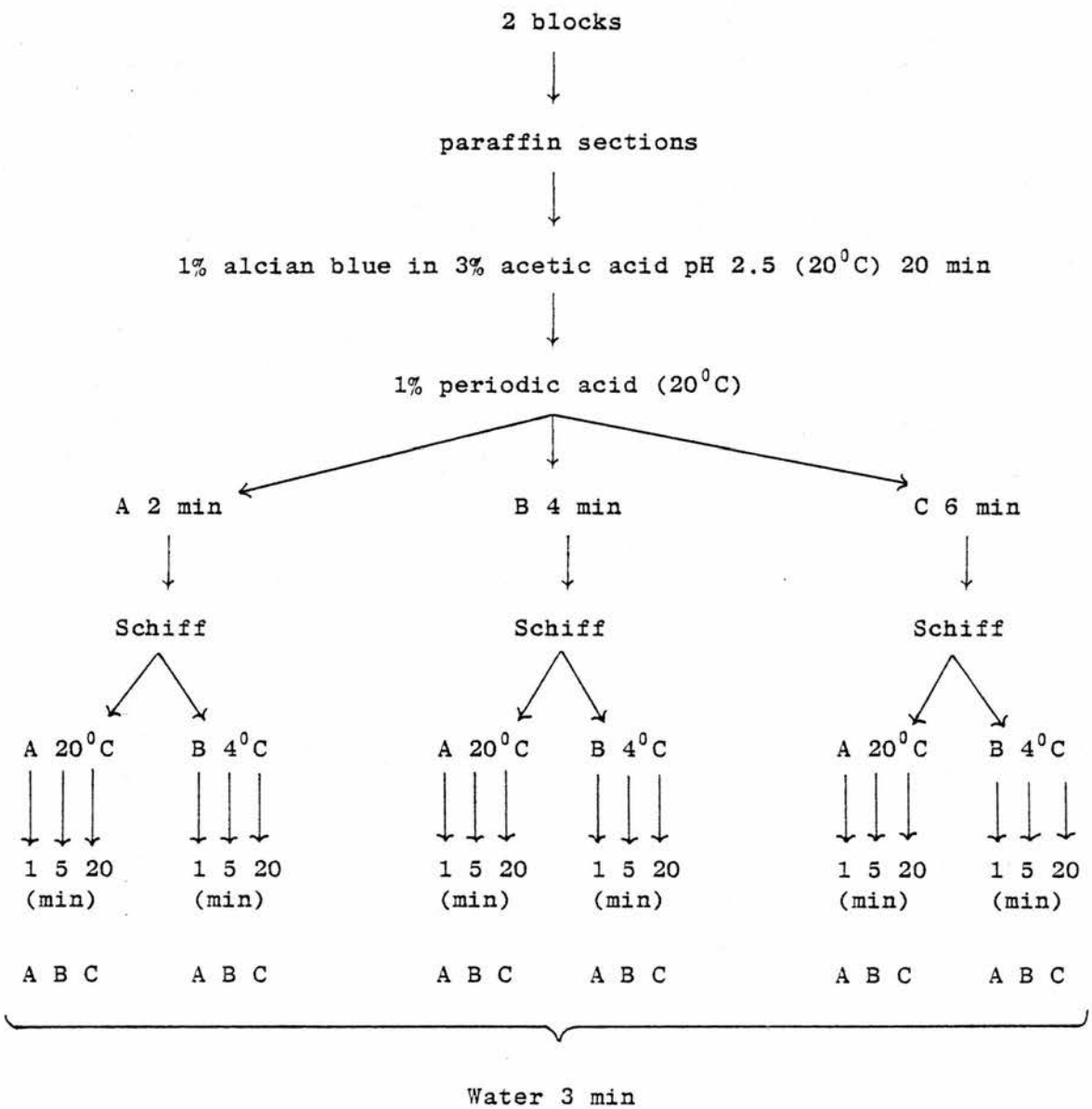
Six blocks of tissue were obtained from a mastectomy specimen fixed for 72 hours in Carson's fixative. Each block was stained and cleared. The tissue was thereafter processed as follows:



The temperature for the Schiff reagent was strictly controlled at 20°C and the periodic acid was made up fresh for the experiment. Six control sections from stained and cleared biopsies, diagnosed as breast fibroadenomata, and known to display good AB/PAS staining according to the schedule routinely used in the biopsy laboratories, were included. The sections obtained showed that the AB/PAS was most satisfactory using a 4 min exposure to periodic acid. A further experiment was performed to establish whether the use of "hot" (20°C) or "cold" (5°C) Schiff affected staining and it was found that the use of Schiff controlled at 20°C was preferable.

To estimate the optimum exposure time to Schiff reagent and to provide confirmation of the correct exposure to periodic acid as well as the temperature of the Schiff to be used in the study, the following trial was performed.

A surgical mastectomy specimen was sliced, stained and cleared. Two blocks of tissue known to contain parenchyma as a result of subgross examination were selected and processed for paraffin wax sections. Sixteen serial sections were cut from each block. The sections, with controls from biopsies of breast fibroadenomata, were coded and put through the schedule below:



The coded slides were assessed blind by two independent observers for quality of staining. The slide of choice was: BAB 1 4 minutes exposure to periodic acid followed by staining in Schiff's reagent at 20^0C for 5 minutes.

The experiment was repeated immediately prior to the main study in order to check that the same result was still selected as optimum. This indeed proved to be the case.

Long Term Effects of Fixation, Staining and Clearing of Tissue

A final experiment was performed to test the long term effects of staining and clearing on tissue as well as the effects of prolonged fixation.

Fresh tissue was obtained from two surgical mastectomy specimens. From each breast:

a) 11 blocks of tissue were placed in Carson's fixative. After 24 hours, 1 block of tissue was processed routinely and 3 paraffin wax sections obtained. One section was stained with H & E; 1 with AB/PAS and 1 was retained as a spare.

b) After 24 hours fixation in Carson's fixative, a second group of 11 blocks of tissue from each breast were stained and cleared using a 24 hour Histokinette schedule. One block of tissue was processed immediately and 3 paraffin sections obtained and stained as for a). The remaining 10 blocks were placed in methyl salicylate for storage.

One cleared and 1 formalin fixed block of tissue from each of the mastectomy specimens were taken from storage at intervals of 1

week for 4 weeks and thereafter every month for 6 months. The tissue was processed and 3 paraffin sections were obtained from each block and stained as for a).

At the end of the trial all the spare sections were stained with AB/PAS in a single batch with sections from fibroadenomata as controls. The sections were coded and examined without prior knowledge of the length of time they had been stored in either fixative or methyl salicylate. It was found that the sections showed a great similarity in AB/PAS staining and it was not possible to determine the period of fixation or the period of time in clearing agent from the staining results. Comparison of AB/PAS sections stained immediately after removal from Carson's fixative and methyl salicylate with the spare sections stained with AB/PAS in one batch at the end of the experiment, confirmed the reproducibility of the staining method.

Diastase Digestion

In order to determine that the diastase digestion technique was functioning satisfactorily and removing glycogen from sections, an experiment was performed to test the effect of increasing concentrations of diastase on tissue known to contain quantities of glycogen.

Blocks of liver tissue were obtained from two cases of sudden death. The two individuals of 12 and 21 years of age had no known history of liver disease. The tissue was processed routinely and 7 paraffin wax sections obtained from each block. One was stained

with H & E, one with AB/PAS and the rest were treated with malt diastase as follows:

0.5% malt diastase	pH 5.5	37^0C	4 hours
0.5% malt diastase	pH 5.5	37^0C	18 hours
1% malt diastase	pH 5.5	37^0C	18 hours
2% malt diastase	pH 5.5	37^0C	18 hours
Distilled water	pH 5.5	37^0C	18 hours for control

After diastase digestion, the sections were stained with AB/PAS.

It was found that, after 4 hours treatment with diastase 0.5%, some glycogen remained. After 18 hours exposure to 0.5, 1 and 2% diastase, all the glycogen was removed and this was confirmed by reference to the H & E and AB/PAS sections without digestion. An 18 hour exposure to 0.5% diastase was selected for the main experiment and liver blocks were used as positive controls.

XIV. PHOTOGRAPHY

Whole Breast Slice and Low Power Subgross Photography

This was performed using two camera stand systems:

a) a Prontor Polaroid MP-3 land camera with F4.5 75 mm lens (Polaroid (UK) Ltd., St. Albans, Herts.).

b) an Olympus OM-1n 35 mm camera with Zuiko auto macro 50 mm F3.5 lens (Gallenkamp Ltd., East Kilbride, Scotland).

Back lighting to the tissue was provided by a Kodak transparency viewer (Kodak Ltd., Glasgow, Scotland).

Subgross Photography (at x 6 - x 50 magnification) and Photomicrography

This was achieved by using a Wild MPS 50 photoautomat camera (Leitz Ltd., Luton, England), with an interchangeable 35 mm magazine and an MPS 55 electronic control unit. Using this system, the image scale in the film plane is calculated from the formula:

Total magnification of microscope x tube factor (1) x camera factor (0.32).

Film

The following Kodak 35 mm colour film types (Kodak Ltd., Glasgow, Scotland), were used:

a) Ektachrome ASA 50 for tungsten light.

b) Ektachrome ASA 400 for daylight.

c) Kodachrome ASA 64.

The following Kodak black and white film types were used:

a) HP5 ASA 400.

b) FP4 ASA 125.

c) Pan F ASA 50.

Printing

Printing was performed on the paper types as listed:-

1. For black and white:

   a) Ilford Ilfobrom 3.IP glossy single weight paper, 12.7 x 17.8 cm (Ilford Ltd., Basildon, Essex).

   b) Ilford Ilfobrom 1B2.IP glossy single weight paper, 20.3 x 25.4 cm.

2. For colour printing, the Ilford Cibachrome-A print system was used.

APPENDIX B

STATISTICS

I. WILCOXON'S SIGNED RANK TEST (Mosteller & Rourke, 1973)

This test compares two populations by pairing the samples. Such pairing cuts down the variability of differences between observations from the two populations and at the same time leaves the average difference unchanged.

Suppose there are n x-measurements and n y-measurements and the null hypothesis that x - y = 0 is to be tested. Then the Wilcoxon statistic, W, is obtained as follows:

1. The absolute differences of the original measurements (xi - yi) are ranked.
2. To the rank of the ith absolute difference attach the sign of xi - yi and denote the signed rank by Ri.
3. Obtain the sum of the signed ranks, W:

$W = R_1 + R_2 + R_3 + \text{----------} R_n$

A large positive sum suggests that x - y = 1;

a large negative sum suggests that x - y = 0.

An example of this test applied to areas of NFC after fixation (x values) and areas of NFC after staining (y values) for the 10 whole breasts in the preliminary study is given below. The figures for area are expressed in cm^2. The purpose of the test was to discover whether there is a significant increase or decrease in the area of NFC recorded after staining. Values for only one of each pair of breasts are included and therefore the number of specimens (n) is 8 rather than 10. The test is reproduced as follows:

x	y	x - y	Rank of (x - y)	Signed ranks Ri
713.20	923.37	-210.17	8	-8
155.04	274.53	-119.49	7	-7
359.46	399.72	- 40.26	4	-4
758.74	656.12	102.62	6	+6
502.77	487.19	15.58	2	+2
662.09	702.86	- 40.77	5	-5
186.18	170.39	15.79	3	+3
112.24	123.59	- 11.35	1	-1

W = 13
W + 11
W - 24

W = Wilcoxon's statistic
n = 8

This result is not significant at the 10% level.

For populations where n is > 25, the expected mean (et) of t must be calculated:

$$et = \frac{n(n + 1)}{4}$$

then the standard deviation (s.d.) of the expected mean calculated:

$$et\ s.d. = \sqrt{\frac{n(n + 1)(2n + 1)}{24}}$$

Thus

$$t = \frac{\text{actual - expected mean}}{\text{s.d.}}$$

The result for t must be related to tables for normal distribution using a two-sided test.

The Wilcoxon statistic represents a move in between the sign test and the direct treatment of observations through the t statistic. The sign test disregards the size of the measurements: differences of 10 and 1000 each rate one + sign. The Wilcoxon test takes modest account of size, but is not sensitive to great differences in the size of x - y unless the sample size is large. Thus the Wilcoxon prevents giving huge weight to wild observations.

II. THE FRIEDMAN STATISTIC (Mosteller & Rourke, 1973)

This test is applied where there are several rankings of the same sorts of item, and testing for the existence of correlation between the rankings is desired. The Friedman statistic ($\chi^2 r$) measures the disagreements between the rank sums of the items. When the null hypothesis of random independent rankings holds, $\chi^2 r$ has approximately a chi-square distribution with I - 1 degrees of freedom.

Given m rankings of I items, the Friedman statistic is:

$$\chi^2 r = \frac{12}{mI\ (I + 1)} \quad \Sigma\ Ri^2 - 3m\ (I + 1)$$

where Ri is the sum of the ranks assigned to item i, i = 1, 2....., I

An example of the use of the Friedman statistic is given below. For the 10 breasts in the preliminary study the results for the percentage of total lobules per quadrant are tabulated.

QUADRANTS

Percentage of total lobules

UIQ	UOQ	LIQ	LOQ
46.17	29.01	17.92	6.90
37.64	9.72	44.77	7.72
31.76	20.36	23.57	24.31
32.65	29.08	20.71	17.57
38.25	40.81	14.12	6.82
30.20	33.85	18.57	17.39
17.31	31.62	9.09	41.96
23.93	34.11	16.99	24.97
14.47	54.74	23.09	7.70
14.07	49.57	12.62	23.74

The rankings for the items from the highest (1) to the lowest (4) percentage in each breast are:

UIQ	UOQ	LIQ	LOQ
1	2	3	4
2	3	1	4
1	4	3	2
1	2	3	4
2	1	3	4
2	1	3	4
3	2	4	1
3	1	4	2
3	1	2	4
3	1	4	2
$R_1 = 21$	$R_2 = 18$	$R_3 = 30$	$R_4 = 31$

where I = 4
m = 10

$$\chi^2 r = \frac{12}{mI\ (I + 1)} \quad \Sigma \quad Ri^2 - 3m\ (I + 1)$$

$$\chi^2 r = \frac{12}{10 \times 4(5)} \quad \Sigma \quad 21^2 + 18^2 + 30^2 + 31^2 - 30\ (4 + 1)$$

$$\chi^2 r = \frac{12}{200} \quad \Sigma \quad 2626 - 150$$

$$\chi^2 = 157.56 - 150$$

$$\chi^2 r = 7.56$$

This result shows that the results for the quadrants are significantly different from each other ($p < 0.04$).

III. THE SPEARMAN RANK CORRELATION COEFFICIENT (Rho (ρ)), (Maxwell, 1967)

A correlation coefficient measures the degree of relation between two variables. The ordinary product - moment correlation coefficient (r) measures the degree to which two variables are linearly related and it is therefore fair to say that the correlation measures the degree to which the points (x,y) cluster around a slanted straight line.

Sometimes the original observations are only orderings in x and orderings in y rather than measurements and cannot be computed. Sometimes also, the choice of scale used for the original measurements is uncertain and makes it desirable to have a measurement that does not depend on the scale, provided that the measurements retain their order. The rank correlation coefficient is one simple measure of correlation that has this variance property.

Definition for the Rank Correlation Coefficient

If a sample of n individuals is ranked twice so that the ith individual has an x-rank xi and a y-rank yi, then the rank correlation

$$\text{is } \rho = 1 - \frac{6 \sum di^2}{n(n^2 - 1)}$$

$$\text{where } di = xi - yi$$

An example of the test applied to the ten breasts quantified in the preliminary study to discover whether there is a correlation between age in years and number of lobules is given below. Only the results for one of each of the two pairs of breasts in the sample were included to prevent bias and therefore n = 8 rather than 10.

The test is reproduced as follows:-

Age in years	xi	No. of lobules	yi	xi - yi	di^2
19	$1\frac{1}{2}$	47,663	2	$-\frac{1}{2}$	0.25
19	$1\frac{1}{2}$	70,829	1	$+\frac{1}{2}$	0.25
22	3	29,102	5	-2	4
26	4	32,801	4	0	0
43	$5\frac{1}{2}$	38,563	3	$+2\frac{1}{2}$	6.25
43	$5\frac{1}{2}$	21,242	7	$-1\frac{1}{2}$	2.25
50	7	22,242	6	1	1
63	8	6,467	8	0	0
					14

n = 8

$$\rho = 1 - \frac{6 \times 14}{8\ (64 - 1)}$$

$$= 0.833$$

This result is significant ($p < 0.02$).

For samples where n is $\geq$ 10, the significance of Rho (ρ) can be tested by referring:

$$t = \rho \sqrt{(n - 2) / (1 - \rho^2)}$$

to the t-distribution with n - 2 degrees of freedom.

IV. THE MANN-WHITNEY U TEST (Siegel, 1956)

The Mann-Whitney U test may be used to test whether two independent groups have been drawn from the same population.

Suppose that there are two populations: population A and population B. The null hypothesis is that A and B have the same distribution. The alternative hypothesis H_1, against which H_0 is tested is that A is statistically larger than B, a directional hypothesis. H_1 may be accepted if the probability that a score from A is larger than a score from B is greater than one-half, i.e. if a is one observation from population A, and b is one observation from population B, then H_1 is that $p(a > b) > \frac{1}{2}$. If the evidence supports H_1, this implies that the "bulk" of population A is higher than the bulk of population B.

It might be predicted instead that B is statistically larger than A. Then H_1 would be that $p(a > b) < \frac{1}{2}$. Confirmation of this assertion would imply that the bulk of B is higher than the bulk of A.

For a two-tailed test, i.e. for a prediction of differences which do not state direction, H_1 would be that $p(a > b) \neq \frac{1}{2}$.

Method

Let n_1 = the number of cases in the smaller of two independent groups, and n_2 = the number of cases in the larger. To apply the U test, the observation or scores from both groups are combined and ranked in order of increasing size. In the ranking, algebraic size is considered, i.e. the lowest ranks are assigned to the largest

negative numbers, if any.

Attention is now focused on one of the groups, e.g. the one with n_1 cases. The value of U is given by the number of times that a score in the group with n_2 cases precedes a score in the group with n_1 cases in the ranking. When U has been found, the value of U for n_2 may be calculated by:

$$U = n_1 n_2 - U^1$$

The smaller figure of U or U^1 (along with the number of cases in n_1 and n_2) is referred to the appropriate tables to determine whether the result is significant.

For samples where n_2 (the size of the larger of the two independent samples) is between 9 and 20, the following may be used:

$$U = n_1 n_2 + \frac{n_1(n_1 + 1)}{2} - R_1$$

or equivalently,

$$U = n_1 n_2 + \frac{n_2(n_2 + 1)}{2} - R_2$$

where R_1 = sum of the ranks assigned to the group whose sample size is n_1.

R_2 = sum of the ranks assigned to the group whose sample size is n_2.

The two formulae yield different U's. It is the smaller of the two that is desired. The larger value is U^1. To check whether U^1 has been found rather than U, the transformation $U = n_1 n_2 - U^1$ may

be applied. The smaller of the two values, U_1 is the one whose sampling distribution is the basis for the tables which test for significance. The simplest method to check that U has been found is to use one of the formulae followed by the transformation.

For samples where n_2 is > 20, the standard tables for Mann-Whitney may not be used. As $n_1 n_2$ increase in size, the sampling distribution of U rapidly approaches the normal distribution, with

$$\text{Mean} = \mu u = \frac{n_1 n_2}{2}$$

$$\text{s.d.} = \mu u = \sqrt{\frac{(n_1)(n_2)\ (n_1 + n_2 + 1)}{12}}$$

Therefore when $n_2 > 20$, the significance of an observed value of U may be determined by

$$z = \frac{U - \mu u}{\sigma u}$$

$$= \frac{U - \frac{n_1 n_2}{2}}{\sqrt{\frac{(n_1)(n_2)\ (n_1 + n_2 + 1)}{12}}}$$

which is practically normally distributed with zero mean and unit variance. It does not matter whether U or U^1 is used for the absolute value of z will be the same if either is used. The sign of z depends on whether U or U^1 is used, but the value does not.

In this project, tied scores between two or more observations in the same group which would have affected the value of U, did not

occur in samples to which this test was applied.

An example of the Mann-Whitney U test is given below:

Out of a total of 72 breast quadrants examined, 12 contained tumour biopsy sites and 60 contained normal tissue. To determine whether the densities of lobules found in the tumour quadrants were significantly higher or lower than those recorded in the normal quadrants, the results were ranked and a Mann-Whitney test performed:

$$n_1 = 12$$

$$n_2 = 60$$

$$R_1 = 376$$

$$U = n_1 n_2 + \frac{n_1(n_1 + 1)}{2} - R_1$$

$$= 720 + \frac{156}{2} - 376$$

$$= 422$$

As $n_2 > 20$,

$$z = \frac{U - \frac{n_1 n_2}{2}}{\sqrt{\frac{n_1 n_2 (n_1 + n_2 + 1)}{12}}}$$

$$= \frac{422 - \frac{720}{2}}{\sqrt{\frac{720 \times 73}{12}}}$$

$$= \frac{62}{66.18}$$

$$= - 0.937$$

This result is not significant and therefore, in terms of lobule density, the tumour quadrants were not different from the normal quadrants. The great advantage of this test is that uneven samples from the same population may be compared.

V. MATHEMATICAL MODEL FOR THE DERIVATION OF THE COEFFICIENT OF VARIATION, APPLIED TO THE PRELIMINARY STUDY ON QUANTITATION

Lobules of the ith slice (L_i) = density x area of the ith slice (A_i) + e_i

where e_i = difference or error term.

It is assumed that SD (L_i) $\alpha \sqrt{A_i}$

where SD = standard deviation.

SD number of lobules $\alpha \sqrt{A_i} = \sigma \sqrt{A_i}$

where σ = constant of proportionality.

Method of Estimating the Effect of Counting only Some Slices

This derivation is a straightforward application of a standard regression theory to this case.

Suppose that the actual number of lobules in the ith slice counted is L_i and the actual number of lobules for the jth slice not counted = L_j:

$$\text{estimated total lobules then} = \Sigma L_i + \Sigma \text{ estimated } L_j$$

$$= \Sigma L_i + \hat{d} \Sigma A_j$$

$$\text{where } \hat{d} = \text{estimated density} = \frac{\Sigma L_i}{\Sigma A_i}$$

ΣA_j = sum of areas of slices not counted.

The sources of error in this estimate are:

i) The differences e_i between each L_j and A_j.

ii) Error in estimating the density from only some of the slices.

This leads to:

$$\text{Variance of estimated lobules} = (SD)^2$$

$$= \Sigma \sigma^2 A_j + \text{var}(\hat{d})(\Sigma A_j)^2$$

$$= \sigma^2 \Sigma A_j + (\Sigma A_j)^2 \left[\frac{\sigma^2}{\Sigma A_i}\right] \qquad (1)$$

Where ΣA_i = sum of the areas counted.

Where $\frac{\sigma^2}{\Sigma A_i}$ = variance of $\hat{d}$

we need an estimate of σ^2

For those that are counted completely, the best estimate of σ^2 is:

$$\hat{\sigma}^2 = \Sigma (L_k - \hat{d} A_k)^2 \frac{1}{A_k} / (n - 1) \qquad (2)$$

where L_k and A_k are all the slices.

From (1): variance of estimated lobules =

$$\hat{\sigma}^2 \Sigma A_j \left[1 + \frac{\Sigma A_j}{\Sigma A_i}\right]$$

Coefficient of variation of estimated lobules $(CV)^2$

$$= \hat{\sigma}^2 \frac{\Sigma A_j}{[\Sigma (A_i + A_j)]^2 \times (\hat{d})^2} \left[1 + \frac{\Sigma A_j}{\Sigma A_i}\right]$$

$$= \frac{\hat{\sigma}^2}{\hat{d}^2 \Sigma (A_i + A_j)} \left[\frac{\Sigma A_j}{\Sigma A_i + A_j}\right] \left[1 + \frac{\Sigma A_j}{\Sigma A_i}\right]$$

$$(CV)^2 = \frac{\Sigma (L_k - \hat{d} A_k)^2 / A_k}{\hat{d}^2 \Sigma (A_i + A_j)(n - 1)} \left[\frac{\Sigma A_j}{\Sigma (A_j + A_i)}\right] \left[1 + \frac{\Sigma A_j}{\Sigma A_i}\right]$$

$$= \frac{(\text{CV of density})^2}{n - 1} \left[\frac{\Sigma A_j}{\Sigma (A_j + A_i)}\right] \left[1 + \frac{\Sigma A_j}{\Sigma A_i}\right] \qquad (3)$$

The square root gives the coefficient of variation of the estimated lobules (as a proportion) when the j slices only are counted. Thus, to estimate the effect of counting the slices which make up half the area, put $\Sigma A_j / \Sigma (A_j + A_i)$ equal to $\frac{1}{2}$ in (3).

This mathematical model and method of estimating the effect of counting a proportion of slices of breast tissue was provided by Mrs G.M. Raab (Medical Computing and Statistics Unit, University of Edinburgh).

APPENDIX C

TABLES

TABLE 1:

DETAILS OF WHOLE BREASTS AND CASE DATA

Age (in yrs)	Side	Parity	Pill	Day of cycle	Tumour quadrant	Family history	Weight in grams	Reference No.
A 19	R				-		307	RF2
A 19	R*				-		430	RF4*
	L*				-		465	LF4*
A 21	R*				-		450	RF5*
	L*				-		350	LF5*
A 22	R*				-		455	RF3*
	L*				-		555	LF3*
A 25	R*				-		300	RF6*
	L*				-		250	LF6*
25	L	0 + 0	+	7	UOQ/LOQ	+	485	8276/80
26	L	0 + 0	+	10	LOQ	-	585	5912/78
29	R	1 + 0	+	19	LOQ	-	350	10701/78
29	R	1 + 0	+	21	LOQ	-	400	989/80
30	R	2 + 0	-	28	UIQ	-	295	7831/80
31	L	2 + 0	-	26	UOQ	-	305	7185/79
34	R	2 + 0	-	14	N	+	360	13937/78
35	L	0 + 0	+	19	N	+	460	7456/79
37	R	4 + 2	-	8	UIQ	-	345	7883/79
38	R	4 + 0	-	H(37)	UOQ	-	780	6824/80
38	L	2 + 0	-	27	LIQ	-	365	189/80
39	L	0 + 0	-	27	LOQ	+	250	165/80
40	R	3 + 0	-	23	LIQ	-	345	8275/80
41	R	2 + 0	-	25	LOQ	-	650	16001/79
41	L	0 + 0	-	8	UOQ	-	805	1322/79
41	L	3 + 2	-	21	LIQ	-	220	14363/78
42	R	2 + 0	-	10	UOQ	-	385	13936/78
43	L	3 + 2	-	M	UIQ	-	300	6441/78
43	L	3 + 0	-	12	UIQ	-	835	6390/78
44	R*	3 + 0	-	2	-	-	315	11519/79
	L*				UOQ		295	
46	R	2 + 0	-	H(37)	-	-	330	4722/79
46	L	1 + 1	-	M	UOQ	-	550	1576/79
46	L	2 + 1	+	M	UOQ	-	250	266/79
46	L	0 + 0	-	24	UOQ	-	1345	6064/80

TABLE 1 (contd.)

Age (in yrs)	Side	Parity	Pill	Day of cycle	Tumour quadrant	Family history	Weight in grams	Reference No.
49	R*	3 + 0	-	1	-	-	415	16599/79
	L*				UOQ		435	
50	L	2 + 0	-	H(46)	LOQ	+	556	12081/78
50	L	4 + 0	-	26	LOQ	-	295	267/79
50	L	1 + 0	-	M	UOQ	+	520	213/80
A 50	R*			M	-		275	381/78
	L*				-		250	
53	L	0 + 0	+	18	UOQ	+	260	1405/80
54	L	6 + 0	-	H(49)	LOQ	-	625	1404/80
55	L	2 + 1	-	H(41)	UOQ	-	300	2818/80
56	R*	3 + 0	-	PM	UOQ	-	485	607/80
	L*				-		350	
57	L	1 + 0	-	H(44)		-	485	15867/78
63	R*	3 + 0	-	H(42)	-	-	250	6176/78
	L*				-		215	
73	L	0 + 0	-	H(44)	UOQ	-	580	990/80

A: autopsy
R: right
L: left
M: menopausal
PM: postmenopausal
N: nipple
H: hysterectomy followed by age in years ( )
* matching pair of right and left breasts
+ currently on the pill/positive family history of carcinoma
- not on the pill/no family history of carcinoma

Total: 50 breasts

9 pairs of specimens

TABLE 2:

LISTING BY REFERENCE NUMBER OF THE 50 BREASTS STUDIED

Age (in yrs)	Preliminary Study	Main Study		Lobule Morphology	Duct Injections
		NFC	Lobules		
50	R381/78	R381/78		R381/78	
63	L6176/78	L6176/78			
63	R6176/78	R6176/78		R6176/78	
26	L5912/78			L5912/78	
43	L6390/78			L6390/78	
43	L6441/78			L6441/78	
19	RF2	RF2		RF2	
19	RF4			RF4	
22	RF3	RF3			
19	LF4			LF4	
41		R1322/79	R1322/79	R1322/79	
42		R13936/78	R13936/78	R13936/78	
57		L15867/78		L15867/78	
37		R7883/79		R7883/79	
46		L1576/79		L1576/79	
41		L14363/78	L14363/78	L14363/78	
46		L266/79		L266/79	
25		L8276/80	L8276/80	L8276/80	L8276/80
22		LF3	LF3	LF3	
38		R6824/80		R6824/80	
35		L7456/79	L7456/79	L7456/79	
30		R7831/80	R7831/80	R7831/80	R7831/80
29		R989/80	R989/80	R989/80	R989/80
53		L1405/80		L1405/80	
73		L990/80		L990/80	
55		L2818/80	L2818/80	L2818/80	
40		R8275/80		R8275/80	R8275/80
46		L6064/80		L6064/80	L6064/80
44		L11519/79		L11519/79	
25		RF6	RF6	RF6	
25		LF6	LF6	LF6	

TABLE 2 (contd.)

Age (in yrs)	Preliminary Study	Main Study		Lobule Morphology	Duct Injections
		NFC	Lobules		
21		RF5	RF5	RF5	
21		LF5	LF5	LF5	
56		R607/80	R607/80	R607/80	
56		L607/80	L607/80	L607/80	L607/80
31			L7185/79	L7185/79	
29			R10701/80	R10701/80	
38			L189/80	L189/80	L189/80
54			L1404/80	L1404/80	L1404/80
39			L165/80	L165/80	L165/80
46				R4722/79	
50				L267/79	
34				R13937/78	
50				L12081/78	
50				L213/80	
44				R11519/79	
49				R16599/79	
49				L16599/79	
50				L381/78	
41					R16001/79

TABLE 3:

DETAILS OF MASTECTOMIES

Age (in yrs)	Size of biopsy in cm	Size in cm of residual tumour in the breast where present	Presence of axillary tail tissue as a separate specimen	Diagnosis of tumour/pathological lesion
25	2.0 x 1.5 x 1.2			Invasive and intraduct carcinoma
26	2.0 x 2.0 x 1.2	1.5 x 0.8 x 0.6		Invasive anaplastic mucoid carcinoma with an area of intraduct carcinoma
29	2 fragments - max. diam. 2.5		+	Invasive adenocarcinoma
29	Trucut	3.2 x 2.5 x 2.0		Infiltrating and intraduct carcinoma
30	4.0 x 4.0 x 2.0			Invasive carcinoma, ductal type
31	6.0 x 4.0 x 2.0			Intraduct and invasive carcinoma
34	1.0 x 1.0 x 1.0		+	Adenocarcinoma, scirrhous type
35	Trucut	2.3 x 2.0 x 1.6		Invasive duct carcinoma of atypical medullary type
37	Trucut			Scirrhous carcinoma
38	1.5 x 1.2 x 1.0			In situ cribriform carcinoma and intraduct carcinoma
38	4.5 x 3.5 x 2.5			Intraduct carcinoma and intraduct papilloma
39	3.0 x 2.5 x 1.0		+	Invasive duct carcinoma
40	3.5 x 3.0 x 1.0			Intraduct carcinoma, lobular carcinoma in situ and invasive carcinoma of mixed tubular and lobular patterns
41	7.0 x 6.0 x 3.5			Invasive intraduct carcinoma
41	Smears	1.6 x 1.2 x 1.4	+	Invasive duct carcinoma with residual intraduct component
41	2.0 x 1.5 x 0.8		+	Small celled carcinoma
42	4.5 x 4.0 x 2.0		+	Intraduct carcinoma with benign mammary dysplasia
43	4.0 x 1.5 x 1.5			Atypical lobular hyperplasia Grade 4 which should be regarded as lobular carcinoma in situ
43	Trucut			Poorly differentiated pleomorphic carcinoma with adjacent intraduct carcinoma

TABLE 3 (contd.)

Age (in yrs)	Size of biopsy in cm	Size in cm of residual tumour in the breast where present	Presence of axillary tail tissue as a separate specimen	Diagnosis of tumour/pathological lesion
44	3.0 x 2.5 x 1.5		+	Left breast biopsy: multifocal invasive lobular carcinoma with sclerotic intralobular component
46	No biopsy			No tumour pathology
46	4.0 x 3.5 x 2.0		+	Intraduct carcinoma with sclerosing adenosis and microcalcification
46	Trucut	2.5 x 2.5 x 1.5		Invasive carcinoma, moderately pleomorphic and intraduct carcinoma of no specific type
49	Trucut			Right breast biopsy: lobular carcinoma in situ
50	3.5 x 3.0 x 0.5			Intraduct carcinoma
50	1.2 x 1.0 x 0.8			Invasive small celled carcinoma
50	1.6 x 1.0 x 1.0		+	Infiltrating carcinoma predominantly ductal with areas of cribriform duct carcinoma and areas of distinct tubular differentiation
53	Trucut	2.0 x 1.0 x 1.0		Invasive ductal carcinoma of no specific type
54	6.5 x 4.0 x 2.5			Invasive duct carcinoma
55	4.0 x 3.5 x 2.0			Infiltrating duct carcinoma and intraduct carcinoma
56	1.8 x 1.0 x 1.5			Invasive lobular carcinoma
57	5.5 x 4.0 x 1.8		+	Invasive lobular carcinoma with a focus of cribriform carcinoma
63	Trucut			Grade 3-4 atypia. No carcinoma
73	Trucut	2.0 x 1.0 x 1.0		Invasive scirrhous carcinoma

TABLE 4:

LOBULE NUMBERS IN 10 HUMAN BREASTS WITH PERCENTAGE DISTRIBUTION IN EACH QUADRANT

Side	Age (in yrs)	No. of lobules	UIQ	UOQ	LIQ	LOQ	cm^2 areas counted	No. of lobules per unit area
R*	19	70.829	46.17	19.01	17.92	6.90	1451	48.854
R	19	47,663	37.62	9.72	44.74	7.92	914	52.115
R	22	29,102	31.76	20.36	23.57	24.31	2214	13.145
R	50	22,242	32.65	29.08	20.70	17.57	789	28.190
R*	63	11,369	38.25	40.81	14.12	6.82	678	16.766
L*	19	72,547	30.20	33.85	18.56	17.39	1657	43.780
L	26	32,801	17.32	31.62	9.09	41.97	1534	21.854
L	43	38,563	23.93	34.11	16.99	24.97	2401	16.060
L	43	21,242	14.47	54.74	23.09	7.70	500	42.480
L*	63	6,467	14.07	49.57	12.62	23.74	563	11.490

* Matching pairs of right and left breasts

TABLE 5:

TOTAL AREA IN CM^2 AND NUMBER OF LOBULES IN THE QUADRANTS OF 10 BREASTS

Side	Age (in yrs)	Quadrant	After fixation	After staining	No. of lobules
R*	19	UOQ	375.73	375.98	20,546
		UIQ	628.63	603.12	32,703
		LOQ	241.42	226.99	4,889
		LIQ	328.23	338.71	12,691
R	19	UOQ	230.87	237.27	4,629
		UIQ	289.63	267.77	17,933
		LOQ	217.05	196.22	3,776
		LIQ	277.56	269.02	21,325
R	22	UOQ	494.67	532.80	5,928
		UIQ	480.36	479.68	9,244
		LOQ	580.29	549.05	7,070
		LIQ	700.67	637.77	6,860
R	50	UOQ	276.65	257.86	6,467
		UIQ	239.87	209.27	7,261
		LOQ	250.42	232.79	3,908
		LIQ	267.76	288.89	4,606
R*	63	UOQ	246.95	250.57	4,640
		UIQ	274.47	254.77	4,349
		LOQ	145.25	139.73	1,605
		LIQ	133.21	134.60	775
L*	19	UOQ	685.24	683.25	24,554
		UIQ	546.89	554.69	21,907
		LOQ	404.26	372.59	12,614
		LIQ	286.61	289.82	13,472
L	26	UOQ	729.63	695.03	10,372
		UIQ	689.88	650.51	5,680
		LOQ	560.49	517.53	13,766
		LIQ	443.62	451.84	2,983
L	43	UOQ	990.58	947.17	13,154
		UIQ	799.40	630.42	9,229
		LOQ	819.40	805.90	9,628
		LIQ	882.10	700.66	6,552
L	43	UOQ	168.31	170.28	11,627
		UIQ	107.21	101.92	3,074
		LOQ	122.30	105.36	1,636
		LIQ	137.54	133.07	4,905
L*		UOQ	347.28	288.66	3,206
		UIQ	120.29	109.58	910
		LOQ	115.44	100.30	1,535
		LIQ	136.60	128.24	816

*Matching pairs of right and left breasts.

TABLE 6:

AREA IN CM^2 OF THE NON-FATTY COMPONENT IN THE QUADRANTS OF 10 BREASTS

Side	Age (yrs)	Quadrant	After fixation	After staining
R*	19	UOQ	228.06	338.66
		UIQ	179.15	247.58
		LOQ	177.77	193.19
		LIQ	128.22	143.94
R	19	UOQ	34.77	23.04
		UIQ	34.28	94.42
		LOQ	33.89	24.30
		LIQ	52.10	132.77
	22	UOQ	42.81	50.45
		UIQ	118.04	122.91
		LOQ	53.93	82.14
		LIQ	144.68	144.22
R	50	UOQ	222.06	206.87
		UIQ	219.00	142.84
		LOQ	160.63	178.57
		LIQ	157.05	127.84
R*	63	UOQ	45.86	62.24
		UIQ	30.61	55.48
		LOQ	22.79	37.99
		LIQ	7.47	14.73
L*	19	UOQ	220.68	245.98
		UIQ	125.80	140.44
		LOQ	256.73	245.90
		LIQ	131.15	128.03
L	26	UOQ	131.46	126.92
		UIQ	36.58	39.74
		LOQ	231.77	213.43
		LIQ	102.96	107.10
L	43	UOQ	196.92	232.62
		UIQ	178.91	160.76
		LOQ	168.14	205.48
		LIQ	118.12	104.00
L	43	UOQ	88.41	89.16
		UIQ	35.62	32.49
		LOQ	29.15	17.91
		LIQ	33.00	30.83
L*	63	UOQ	41.39	54.73
		UIQ	19.49	13.91
		LOQ	32.64	36.94
		LIQ	18.72	18.01

*Matching pairs of right and left breasts.

TABLE 7:

PERCENTAGE OF THE NON-FATTY COMPONENT AND FAT IN THE QUADRANTS OF 10 BREASTS

Side	Age (yrs)	Quadrant	After Fixation		After Staining	
			% NFC	% Fat	% NFC	% Fat
R*	19	UOQ	60.70	37.30	90.07	9.93
		UIQ	28.50	71.50	41.50	58.95
		LOQ	73.64	39.30	85.11	14.89
		LIQ	39.60	60.94	42.50	57.50
R	19	UOQ	15.60	84.94	9.71	90.29
		UIQ	11.84	88.16	35.26	64.74
		LOQ	15.61	84.39	12.38	87.62
		LIQ	18.77	81.23	49.35	50.65
R	22	UOQ	8.66	91.34	9.47	90.53
		UIQ	24.57	75.43	25.62	74.38
		LOQ	9.30	90.70	15.20	84.98
		LIQ	20.65	79.35	22.62	77.38
R	50	UOQ	80.27	19.73	80.23	19.77
		UIQ	91.30	8.70	68.26	31.74
		LOQ	64.14	35.86	76.71	23.29
		LIQ	58.65	41.35	68.26	44.15
R*	63	UOQ	18.57	81.43	24.85	75.15
		UIQ	11.15	88.85	21.78	78.22
		LOQ	15.69	84.31	27.00	73.00
		LIQ	5.60	94.40	10.94	89.06
L*	19	UOQ	32.20	67.80	36.00	64.00
		UIQ	23.00	77.00	25.32	74.68
		LOQ	63.51	36.49	66.00	34.00
		LIQ	45.76	54.24	44.18	55.82
L	26	UOQ	18.02	81.98	18.26	81.74
		UIQ	5.30	94.70	6.11	93.85
		LOQ	41.21	58.79	41.24	58.76
		LIQ	23.21	76.75	23.70	76.30
L	43	UOQ	52.53	47.47	52.36	47.64
		UIQ	33.22	66.78	31.88	68.12
		LOQ	23.83	76.17	17.00	83.00
		LIQ	23.99	76.01	23.17	76.83
L	43	UOQ	19.77	80.23	24.56	75.44
		UIQ	22.38	77.62	25.50	74.50
		LOQ	20.52	79.48	25.50	74.50
		LIQ	13.39	86.61	14.84	85.16
L*	63	UOQ	11.92	88.08	18.96	81.04
		UIQ	16.20	83.90	12.70	94.07
		LOQ	28.27	71.73	36.83	63.17
		LIQ	13.71	86.29	14.40	85.60

*Matching pairs of right and left breasts.

TABLE 8:

CORRELATION COEFFICIENTS AND COEFFICIENTS OF VARIATION FOR NUMBERS OF LOBULES AND TOTAL AREAS OF TISSUE FROM CONSECUTIVE SLICES FOR EACH QUADRANT

Right breasts after fixation (age in yrs)	Quadrant	No. of slices	No. of lobules	Density lobules/ cm^2 tissue	Correlation coefficient	Coefficient of variation if 50% slices counted
19	UOQ	15	17,643	66.52	0.98	5.41
	UIQ	15	32,372	61.74	0.86	16.36
	LOQ	16	3,312	17.08	0.85	17.69
	LIQ	14	11,528	44.57	0.93	10.97
19	UOQ	15	4,598	23.09	0.90	14.02
	UIQ	15	14,753	56.77	0.98	5.69
	LOQ	14	3,686	20.62	0.57	41.26
	LIQ	14	18,259	78.47	0.98	5.02
22	UOQ	11	5,958	16.15	0.93	12.87
	UIQ	11	7,353	19.42	0.92	13.96
	LOQ	11	6,499	15.11	0.90	16.31
	LIQ	11	5,950	11.61	0.94	11.88
50	UOQ	8	7,052	32.64	0.98	8.41
	UIQ	5	4,455	28.81	0.93	21.89
	LOQ	8	3,525	16.71	0.81	29.14
	LIQ	5	3,879	20.65	0.98	10.03
63	UOQ	6	4,739	24.62	0.85	26.85
	UIQ	6	3,773	18.80	0.87	27.89
	LOQ	7	600	5.02	0.90	20.82
	LIQ	7	1,025	12.00	0.93	18.86

TABLE 9:

CORRELATION COEFFICIENTS AND COEFFICIENTS OF VARIATION FOR NUMBERS OF LOBULES AND TOTAL AREAS OF TISSUE FROM CONSECUTIVE SLICES FOR EACH QUADRANT

Left breasts after fixation (age in yrs)	Quadrant	No. of slices	No. of lobules	Density lobules/ cm^2 tissue	Correlation coefficient	Coefficient of variation if 50% slices counted
19	UOQ	16	23,306	38.86	0.83	17.75
	UIQ	16	20,589	44.24	0.93	10.67
	LOQ	16	9,320	30.89	0.89	13.71
	LIQ	16	13,388	52.72	0.88	14.57
26	UOQ	16	10,591	16.82	0.90	13.02
	UIQ	16	5,609	9.92	0.90	13.17
	LOQ	16	12,427	28.68	0.87	14.82
	LIQ	16	3,117	8.25	0.83	18.00
43	UOQ	17	12,413	15.55	0.94	9.47
	UIQ	18	9,601	14.84	0.93	11.94
	LOQ	17	8,096	12.15	0.90	9.84
	LIQ	18	5,879	8.23	0.86	14.65
43	UOQ	4	11,018	85.52	0.93	26.20
	UIQ	4	2,807	31.23	0.82	46.79
	LOQ	4	1,640	16.38	0.83	44.79
	LIQ	4	4,469	40.38	0.96	40.06
63	UOQ	7	1,835	6.81	0.79	34.42
	UIQ	7	1,160	12.16	0.89	23.14
	LOQ	7	1,365	18.21	0.95	13.83
	LIQ	7	662	6.00	0.67	48.59

TABLE 10:

CORRELATION COEFFICIENTS AND COEFFICIENTS OF VARIATION FOR NUMBERS OF LOBULES AND TOTAL AREAS OF TISSUE FROM CONSECUTIVE SLICES FOR EACH QUADRANT

Right breasts after staining (age in yrs)	Quadrant	No. of slices	No. of lobules	Density lobules/ cm^2 tissue	Correlation coefficient	Coefficient of variation if 50% slices counted
19	UOQ	15	17,643	68.66	0.98	5.73
	UIQ	15	32,372	64.15	0.87	15.70
	LOQ	16	3,312	17.64	0.84	18.59
	LIQ	14	11,528	41.72	0.91	12.31
19	UOQ	15	4,598	22.66	0.91	13.43
	UIQ	15	14,753	63.77	0.98	5.25
	LOQ	14	3,686	22.34	0.60	38.54
	LIQ	14	18,259	80.95	0.99	4.14
22	UOQ	11	5,958	14.35	0.91	15.27
	UIQ	11	7,353	19.25	0.93	12.87
	LOQ	11	6,499	15.15	0.91	15.71
	LIQ	11	5,950	12.82	0.94	11.89
50	UOQ	8	7,052	25.89	0.98	9.36
	UIQ	5	4,455	33.44	0.93	22.27
	LOQ	8	3,525	25.23	0.83	30.74
	LIQ	5	3,879	24.67	0.98	14.68
63	UOQ	6	4,739	23.96	0.86	26.24
	UIQ	6	3,773	18.47	0.89	25.52
	LOQ	7	600	5.16	0.90	21.05
	LIQ	7	1,025	13.18	0.96	15.20

TABLE 11:

CORRELATION COEFFICIENTS AND COEFFICIENTS OF VARIATION FOR NUMBERS OF LOBULES

AND TOTAL AREAS OF TISSUE FROM CONSECUTIVE SLICES FOR EACH QUADRANT

Left breasts after staining (age in yrs)	Quadrant	No. of slices	No. of lobules	Density lobules/ cm^2 tissue	Correlation coefficient	Coefficient of variation if 50% slices counted
19	UOQ	16	23,306	39.12	0.84	17.52
	UIQ	16	20,589	43.44	0.93	10.49
	LOQ	16	9,320	32.36	0.88	14.42
	LIQ	16	13,388	54.03	0.89	13.95
26	UOQ	16	10,591	17.88	0.88	14.14
	UIQ	16	5,609	10.69	0.89	13.79
	LOQ	16	12,427	30.97	0.86	15.68
	LIQ	16	3,117	8.12	0.81	18.99
43	UOQ	17	12,413	15.70	0.95	8.28
	UIQ	15	7,424	14.35	0.94	12.39
	LOQ	17	8,096	12.15	0.90	9.95
	LIQ	17	4,929	8.63	0.88	13.65
43	UOQ	4	11,018	86.40	0.75	20.08
	UIQ	4	2,807	32.25	0.82	46.01
	LOQ	4	1,640	18.04	0.82	46.54
	LIQ	4	4,469	41.39	0.96	18.51
63	UOQ	7	1,835	7.90	0.78	35.72
	UIQ	7	1,160	12.82	0.89	22.20
	LOQ	7	1,365	20.87	0.94	15.87
	LIQ	7	662	6.41	0.68	47.33

TABLE 12:

LOBULE SCORES WHICH ESTIMATE LOBULE NUMBER IN THE FOUR QUADRANTS OF 20 BREASTS

Side	Age (yrs)	UOQ	UIQ	LOQ	LIQ	Total
R*	21	482.5	84.5	227.5	111	905.5
L*	21	177.5	64	334.5	110.5	686.5
L	22	474	439.5	224	294	1431.5
L	25	1059.5	920	487.5	401.5	2868.5
R*	25	1285	674	450.5	135.5	2545
L*	25	274.5	358.5	346	361	1340
R	29	715	363.5	215.5	151	1445
R	29	731.5	683	193.5	302	1910
R	30	434.5	275	354	274	1337.5
L	31	230.5	203	93.5	191	718
L	35	690.5	333.5	392	268	1684
L	38	190	354.5	186	500.5	1231
L	39	119	150	108.5	88	465.5
L	41	353	269.5	156.5	452.5	1231.5
R	41	264	290	296.5	168.5	1019
R	42	74	216.5	143.5	155	589
R	54	148	233.5	107	220.5	709
L	55	53	54	48	52	207
R*	56	245	243.5	137.5	204	830
L*	56	468.5	367.5	459	353	1648

R: Right L: Left *Pairs of Right and Left Breasts.

TABLE 13:

NUMBER OF CM^2 CELLS ASSESSED IN THE FOUR QUADRANTS OF 20 BREASTS

Side	Age (yrs)	UOQ	UIQ	LOQ	LIQ	Total
R*	21	648	131.5	315	214.5	1309
L*	21	257.5	194	553	402	1406.5
L	22	804	643	402.5	570	2419.5
L	25	481	432.5	375	303.5	1592
R*	25	508.5	277.5	164	92	1042
L*	25	222	165	257	180.5	824.5
R	29	642	388	266	155.5	1451.5
R	29	435	403	192.5	196	1226.5
R	30	293.5	209	164.5	151	818
L	31	404.5	385	211	263	1263.5
L	35	900	526	597	337.5	2360.5
L	38	294.5	534.5	165.5	330.5	1325
L	39	106	131.5	67	73.5	378
L	41	221	196.5	122	280	819.5
R	41	805	952	491.5	447.5	2696
R	42	355.5	459.5	398	377	1590
R	54	534	472	552	539	2097
L	55	366	308	142	131.5	947.5
R*	56	285	239.5	186	179	889.5
L*	56	264	221	245.5	221	951.5

R: Right L: Left *Pairs of Right and Left Breasts.

TABLE 14:

AVERAGE LOBULE SCORES PER CM^2 CELL IN THE FOUR QUADRANTS OF 20 BREASTS

Side	Age	UOQ	ULQ	LOQ	LIQ
R	21	0.745	0.643	0.722	0.517
L	21	0.689	0.330	0.605	0.275
L	22	0.590	0.684	0.557	0.516
L	25	2.203	2.127	1.300	1.323
R*	25	2.527	2.429	2.747	1.473
L*	25	1.236	2.173	1.346	2.0
R	29	1.114	0.937	0.810	0.971
R	29	1.682	1.695	1.005	1.541
R	30	1.480	1.316	2.152	1.815
L	31	0.570	0.527	0.443	0.726
L	35	0.767	0.634	0.657	0.710
L	38	0.645	0.663	1.124	1.514
L	39	1.123	1.141	1.619	1.197
L	41	1.594	1.372	1.283	1.616
R	41	0.328	0.305	0.603	0.377
R	42	0.208	0.471	0.361	0.411
R	54	0.277	0.495	0.194	0.409
L	55	0.145	0.175	0.338	0.395
R*	56	0.860	1.017	0.739	1.140
L*	56	1.775	1.663	1.870	1.597

R: Right L: Left *Pairs of Right and Left Breasts.

TABLE 15:

LOBULE SCORES WHICH ESTIMATE LOBULE NUMBERS IN SELECTED AREAS OF 20 BREASTS

Side	Age (yrs)	Outer Half	Inner Half	Upper Half	Lower Half	UOQ + LIQ	UIQ + LOQ
R*	21	710	195.5	567	338.5	593.5	312
L*	21	512	174.5	241.5	472	288	398.5
L	22	698	733.5	913.5	518	768	663.5
L	25	1547	1321.5	1979.5	889	1461	1407.5
R*	25	1735.5	809.5	1959	586	1420.5	1124.5
L*	25	620.5	719.5	633	707	635.5	704.5
R	29	930.5	514.5	1078.5	366.5	866	579
R	29	925	985	1414.5	495.5	1033.5	876.5
R	30	788.5	548	709.5	628	708.5	629
L	31	324	394	433.5	284.5	421.5	296.5
L	35	1082.5	601.5	1024	660	958.5	725.5
L	38	376	855	544.4	872.5	690.5	540.5
L	39	227.5	238	269	196.5	207	258.5
L	41	509.5	722	622.5	609	649	582.5
R	41	560.5	458.5	554	465	432.5	586.5
R	42	217.5	371.5	290.5	298.5	229	360
R	54	255	454	381.5	327.5	368.5	340.5
L	55	101	106	107	100	105	102
R*	56	382.5	447.5	488.5	341.5	449	381
L*	56	927.5	720.5	836	812	821.5	826.5

R: Right L: Left *Pairs of Right and Left Breasts.

TABLE 16:

NUMBER OF CM^2 CELLS ASSESSED IN THE SELECTED AREAS

OF THE 20 BREASTS DESCRIBED IN TABLE 15

Side	Age (yrs)	Outer Half	Inner Half	Upper Half	Lower Half	UOQ + LIQ	UIQ + LOQ
R*	21	963	346	779.5	529.5	862.5	446.5
L*	21	810.5	596	451.5	955	659.5	747
L	22	1206.5	1213	1447	972.5	1374	1045.5
L	25	856	736	913.5	678.5	784.5	807.5
R*	25	672.5	369.5	786	256	600.5	441.5
L*	25	479	345.5	387	437.5	402.5	422
R	29	908	543.5	1030	421.5	797.5	654
R	29	627.5	599	838	388.5	631	595.5
R	30	458	360	502.5	315.5	444.5	373.5
L	31	615.5	648	789.5	474	667.5	596
L	35	1497	863.5	1426	934.5	1237.5	1123
L	38	460	865	829	496	625	700
L	39	173	205	237.5	140.5	179.5	198.5
L	41	343	476.5	417.5	402	501	318.5
R	41	1296.5	399.5	1757	939	1252.5	1443.5
R	42	753.5	836.5	815	775	732.5	857.5
R	54	1086	1011	1006	1091	1073	1024
L	55	508	439.5	674	273.5	497.5	450
R*	56	471	418.5	524.5	365	464	425.5
L*	56	509.5	442	485	466.5	485	466.5

R: Right L: Left *Pairs of Right and Left Breasts.

TABLE 17:

AVERAGE LOBULE SCORES PER CM^2 CELL IN THE SELECTED AREAS

OF THE 20 BREASTS DESCRIBED IN TABLE 15

Side	Age (yrs)	Outer Half	Inner Half	Upper Half	Lower Half	UOQ + LIQ	UIQ + LOQ
R*	21	0.737	0.565	0.727	0.639	0.688	0.699
L*	21	0.632	0.293	0.535	0.494	0.437	0.533
L	22	0.579	0.605	0.631	0.533	0.559	0.635
L	25	1.807	1.796	2.167	1.310	1.862	1.743
R*	25	2.581	2.191	2.492	2.289	2.366	2.547
L*	25	1.295	2.082	1.636	1.616	1.579	1.669
R	29	1.025	0.947	1.047	0.870	1.086	0.885
R	29	1.474	1.644	1.688	1.275	1.638	1.472
R	30	1.722	1.525	1.412	1.990	1.594	1.684
L	31	0.526	0.608	0.549	0.600	0.631	0.497
L	35	0.723	0.697	0.718	0.706	0.775	0.646
L	38	0.817	0.988	0.657	1.759	1.105	0.772
L	39	1.315	1.161	1.133	1.399	1.153	1.302
L	41	1.485	1.515	1.491	1.515	1.295	1.829
R	41	0.432	1.148	0.315	0.495	0.345	0.406
R	42	0.289	0.444	0.001	0.385	0.313	0.420
R	54	0.235	0.450	0.379	0.300	0.343	0.333
L	55	0.199	0.241	0.159	0.366	0.211	0.227
R*	56	0.812	1.069	0.931	0.936	0.968	0.895
L*	56	1.820	1.630	1.724	1.741	1.694	1.772

R: Right L: Left *Pairs of Right and Left Breasts

TABLE 18:

LOBULE SCORES WHICH ESTIMATE LOBULE NUMBERS IN THE SUPERFICIAL, MIDDLE AND DEEP THIRDS OF 19 BREASTS

Side	Age (yrs)	Superficial Third	Middle Third	Deep Third	Total
R	21	309	375.5	221	905.5
L	22	309	721	401.5	1431.5
L	25	784.5	1035.5	1048.5	2868.5
R*	25	781	1009.5	755	2545.5
L*	25	220	700.5	419.5	1340
R	29	517	529.5	398.5	1445
R	29	298	688.5	923.5	1910
R	30	62.5	594.5	680.5	1337.5
L	31	67.5	263.5	387	718
L	35	329	614	741	1684
L	38	422	540	269	1231
L	39	169.5	233	63	465.5
L	41	148	490.5	593	1231.5
R	41	188	484.5	346.5	1019
R	42	140	269	180	589
R	54	199.5	364.5	145	709
L	55	32	81.5	93.5	207
R*	56	276.5	323	230.5	830
L*	56	453.5	780.5	414	1648

R: Right L: Left *Pairs of Right and Left Breasts.

TABLE 19:

NUMBER OF CM^2 CELLS ASSESSED IN THE SUPERFICIAL, MIDDLE AND DEEP THIRD OF THE 19 BREASTS DESCRIBED IN TABLE 18

Side	Age (yrs)	Superficial Third	Middle Third	Deep Third	Total
R	21	465	472	372	1309
L	22	493	914	1012.5	2419.5
L	25	462	535	595	1592
R*	25	348	354	340	1042
L*	25	174	377.5	273	824.5
R	29	538	488	425.5	1451.5
R	29	336	404	486.5	1226.5
R	30	136	415.5	266.5	818
L	31	195.5	484	584	1263.5
L	35	594	786	980.5	2360.5
L	38	390.5	466.5	468	1325
L	39	154	130	94	378
L	41	166.5	290	363	819.5
R	41	638	993.5	1064.5	2696
R	42	227.5	492.5	870	1590
R	54	593	784.5	719.5	2097
L	55	242	316.5	389	947.5
R*	56	249	342	298.5	889.5
L*	56	295	382	274.5	951.5

R: Right L: Left *Pairs of Right and Left Breasts

TABLE 20:

AVERAGE LOBULE SCORES PER CM^2 CELL IN THE SUPERFICIAL, MIDDLE AND DEEP THIRDS OF THE 19 BREASTS DESCRIBED IN TABLE 18

Side	Age (in yrs)	Superficial Third	Middle Third	Deep Third
R	21	0.665	0.780	0.594
L	22	0.627	0.789	0.397
L	25	1.698	1.936	1.762
R*	25	2.244	2.850	2.221
L*	25	1.264	1.856	1.537
R	29	0.961	1.086	0.937
R	29	0.887	1.704	1.898
R	30	0.460	1.431	2.553
L	31	0.345	0.544	0.800
L	35	0.554	0.781	0.756
L	38	1.081	1.158	0.575
L	39	1.101	1.792	0.670
L	41	0.889	1.691	1.634
R	41	0.295	0.488	0.326
R	42	0.615	0.546	0.207
R	54	0.336	0.465	0.202
L	55	0.132	0.258	0.240
R*	56	1.110	0.944	0.772
L*	56	1.537	2.043	1.508

R: Right L: Left *Pairs of Right and Left Breasts

TABLE 21:

STATISTICAL ANALYSES OF THE LOBULE SCORE RESULTS GIVEN IN TABLES 12, 15 and 18

Lobule scores	20 breasts	p	17 breasts	p
UOQ > UIQ	13	not sig.	11	not sig.
UOQ > LOQ	15	< 0.01	14	< 0.01
UIQ > LOQ	14	nearly sig. < 0.06	13	< 0.05
LOQ > LIQ	9	not sig.	8	not sig.
Outer half > inner half	10	not sig.	7	not sig.
Upper half > lower half	16	< 0.01	15	< 0.01
UOQ + LIQ > UIQ + LOQ	14	nearly sig. < 0.06	14	< 0.05

TABLE 22:

STATISTICAL ANALYSES OF THE LOBULE QUANTITATION RESULTS

GIVEN IN TABLES 18-20

	19 breasts	p	17 breasts	p
Lobule scores				
Superficial third < middle third	19	< 0.01	17	< 0.01
Superficial third < deep third	11	< 0.05	11	< 0.05
Middle third > deep third	12	< 0.05	10	not sig.
Average scores per cm^2 cell tissue				
Superficial third < middle third	17	< 0.01	15	< 0.01
Superficial third < deep third	9	not sig.	8	not sig.
Middle third > deep third	15	< 0.05	14	< 0.05
Number of cm^2 cells tissue quantified				
Superficial third < middle third	17	< 0.01	15	< 0.01
Superficial third < deep third	14	< 0.01	13	< 0.01
Middle third > deep third	9	not sig.	6	not sig.

TABLE 23:

AREAS IN CM^2 OF THE NON-FATTY COMPONENT, THE TOTAL AREA AND THE PERCENTAGE OF THE TOTAL AREA OF TISSUE OCCUPIED BY THE NON-FATTY COMPONENT FOR 30 BREASTS

Side	Age	Total NFC	Total Area	% NFC
L	19	896.42	2012.29	44.55
R	19	325.17	1037.79	31.33
R*	21	991.91	1519.70	65.26
L*	21	689.67	1234.62	55.86
R*	22	390.65	2310.08	16.91
L*	22	503.48	2311.86	21.78
L	25	676.64	1526.78	44.32
R*	25	744.81	1063.47	70.04
L*	25	427.75	705.52	60.63
R	29	907.19	1508.32	60.15
R	30	203.47	858.18	23.72
L	35	1210.83	2382.50	50.82
R	37	1186.32	2053.66	57.77
R	38	470.47	2970.74	15.84
R	40	442.85	792.98	55.85
R	41	811.94	2708.01	29.98
L	41	399.19	779.54	51.21
R	42	687.27	1582.13	43.44
L	44	391.72	1288.51	30.40
L	46	379.59	745.83	50.33
L	46	442.37	1301.55	33.99
L	46	1094.38	3670.52	29.82
L	53	363.85	886.22	41.06
L	55	206.86	923.67	22.40
R*	56	586.49	800.94	73.23
L*	56	750.85	982.28	76.44
L	57	184.69	1914.33	9.60
R*	63	98.49	872.79	11.28
L*	63	184.42	766.76	24.05
L	73	370.00	1847.56	20.03

R: Right L: Left *Pairs of Right and Left Breasts.

TABLE 24:

AREA OF THE NON-FATTY COMPONENT IN CM^2 IN THE FOUR QUADRANTS OF 30 BREASTS

Side	Age	UOQ	UIQ	LOQ	LIQ	Total
L	19	304.30	185.92	247.72	158.48	896.42
R	19	33.61	111.12	34.38	146.06	325.17
R*	21	510.34	124.43	258.68	98.46	991.91
L*	21	269.98	142.71	172.77	104.21	689.67
R*	22	59.97	111.23	78.55	140.90	390.65
L*	22	142.56	173.01	85.84	102.07	503.48
L	25	261.70	185.42	132.49	97.03	676.64
R*	25	356.01	168.08	129.19	91.53	744.81
L*	25	101.73	100.02	118.96	107.04	427.75
R	29	352.76	196.68	109.95	247.80	907.19
R	30	63.90	48.53	50.65	40.39	203.47
L	35	532.52	240.58	251.84	185.89	1210.83
R	37	531.51	357.85	145.77	151.19	1186.32
R	38	171.10	153.19	96.28	49.90	470.47
R	40	93.04	78.75	119.76	151.30	442.85
R	41	195.23	186.57	290.49	139.65	811.94
L	41	127.67	82.43	44.03	145.06	399.19
R	42	170.10	195.75	154.07	167.35	687.27
L	44	85.96	132.14	94.19	79.43	391.72
L	46	120.14	12.06	156.59	90.80	379.59
L	46	229.40	125.79	59.83	27.35	442.37
L	46	510.89	287.34	229.11	67.04	1094.38
L	53	145.31	92.93	86.25	39.36	363.85
L	55	68.34	68.73	39.35	32.44	208.86
R*	56	211.15	147.25	115.53	112.56	586.49
L*	56	229.28	196.97	177.75	146.85	750.85
L	57	59.93	29.21	60.82	34.73	184.69
R*	63	25.66	51.40	6.95	14.48	98.49
L*	63	60.65	34.94	52.65	36.18	184.42
L	73	158.30	108.86	81.23	21.61	370.00

R: Right L: Left *Pairs of Right and Left Breasts

TABLE 25:

TOTAL AREA OF TISSUE IN CM^2 IN THE 4 QUADRANTS

OF 30 BREASTS

Side	Age	UOQ	UIQ	LOQ	LIQ	Total
L	19	752.88	573.10	388.35	297.96	2012.29
R	19	237.39	296.57	217.17	286.66	1037.79
R*	21	713.97	276.45	364.52	164.76	1519.70
L*	21	485.32	363.97	217.47	167.86	1234.62
R*	22	522.80	491.34	594.29	701.65	2310.08
L*	22	731.18	642.86	376.76	561.06	2311.86
L	25	468.97	415.92	345.14	296.75	1526.78
R*	25	480.10	271.55	159.88	151.94	1063.47
L*	25	201.01	157.72	196.12	150.67	705.52
R	29	654.26	416.01	280.79	157.26	1508.32
R	30	234.72	302.75	166.24	154.47	858.18
L	35	905.50	487.32	583.96	405.72	2382.50
R	37	749.35	619.16	282.73	402.42	2053.66
R	38	1302.64	780.59	576.63	310.88	2970.74
R	40	201.76	210.12	164.46	216.64	792.98
R	41	729.03	951.73	523.16	504.09	2708.01
L	41	189.35	193.60	116.88	279.71	779.54
R	42	395.12	463.21	377.26	346.54	1582.13
L	44	290.54	374.06	303.63	320.28	1288.51
L	46	286.33	76.02	208.08	175.40	745.83
L	46	229.40	548.36	296.28	227.51	1301.55
L	46	1515.84	1230.65	461.93	462.10	3670.52
L	53	372.84	233.57	156.60	123.21	886.22
L	55	349.11	315.09	147.14	112.33	923.67
R*	56	282.53	205.16	163.02	150.23	800.94
L*	56	263.32	249.73	241.56	227.67	982.28
L	57	668.41	484.10	464.54	297.28	1914.33
R*	63	267.62	304.77	157.85	142.55	872.79
L*	63	372.58	131.00	126.13	137.05	766.76
L	73	796.38	534.77	375.98	140.43	1847.56

R: Right L: Left *Pairs of Right and Left Breasts.

TABLE 26:

PERCENTAGE OF THE TOTAL AREA OF TISSUE OCCUPIED BY THE NON-FATTY COMPONENT IN THE 4 QUADRANTS OF 30 BREASTS

Side	Age	UOQ	UIQ	LOQ	LIQ
L	19	40.42	32.44	63.79	53.19
R	19	14.16	37.47	15.83	50.95
R*	21	71.48	45.01	70.97	59.76
L*	21	55.63	39.21	79.45	62.08
R*	22	11.47	22.64	13.22	20.08
L*	22	19.50	26.91	22.78	18.19
L	25	55.80	50.48	43.05	32.70
R*	25	74.15	61.90	80.80	60.24
L*	25	50.61	63.41	60.65	71.03
R	29	53.92	47.28	39.16	34.83
R	30	27.22	16.05	30.47	26.15
L	35	58.81	49.37	43.13	45.82
R	37	70.93	57.80	51.56	37.57
R	38	13.14	19.62	16.70	16.05
R	40	44.25	35.66	61.48	60.96
R	41	26.78	19.60	55.53	27.70
L	41	67.43	42.58	37.67	51.86
R	42	43.05	42.26	40.84	48.29
L	44	29.59	35.33	31.02	24.80
L	46	42.0	15.87	75.25	51.77
L	46	30.71	22.94	20.19	12.02
L	46	33.70	23.35	49.28	14.51
L	53	38.97	39.79	55.07	31.95
L	55	19.58	21.81	26.74	28.88
R*	56	74.74	71.77	70.87	74.92
L*	56	87.07	78.87	73.59	64.50
L	57	89.67	6.03	13.09	11.68
R*	63	9.04	16.86	4.40	10.16
L*	63	16.28	26.67	41.74	26.40
L	73	19.88	20.36	21.61	15.39

R: Right L: Left *Pairs of Right and Left Breasts.

TABLE 27:

AREA IN CM^2 OF THE NON-FATTY COMPONENT AND THE TOTAL AREA OF TISSUE IN THE OUTER AND INNER HALVES OF 30 BREASTS

		Outer Half		Inner Half	
Side	Age	NFC	Total area	NFC	Total area
L	19	552.02	1141.23	344.40	871.06
R	19	67.99	454.56	257.18	583.23
R*	21	769.02	1078.49	222.89	441.21
L*	21	442.75	702.79	246.92	531.83
R*	22	138.52	1117.09	252.13	1192.99
L*	22	228.40	1107.94	275.08	1203.92
L	25	394.19	814.11	282.45	712.67
R*	25	485.20	639.98	259.61	423.49
L*	25	220.69	397.13	207.06	308.39
R	29	462.71	935.05	444.48	573.27
R	30	114.55	400.96	88.92	457.22
L	35	784.36	1489.46	426.47	893.04
R	37	677.28	1032.08	509.04	1021.58
R	38	267.38	1879.27	203.09	1091.47
R	40	212.80	366.22	230.05	426.76
R	41	485.72	1252.19	326.22	1455.82
L	41	171.70	306.23	227.49	473.31
R	42	324.17	772.38	363.10	809.75
L	44	180.15	594.17	211.57	694.34
L	46	276.73	494.41	102.86	251.42
L	46	289.23	821.96	153.14	479.59
L	46	740.00	1977.77	354.38	1692.75
L	53	231.56	529.44	132.29	356.78
L	55	107.69	496.25	101.17	427.42
R*	56	326.68	445.53	259.81	355.41
L*	56	407.03	504.88	343.82	477.40
L	57	120.75	1132.95	63.94	781.38
R*	63	32.61	425.47	65.88	447.32
L*	63	113.30	498.72	71.12	268.04
L	73	239.53	1172.36	130.47	675.20

R: Right L: Left *Pairs of Right and Left Breasts

TABLE 28:

AREA IN CM^2 OF THE NON-FATTY COMPONENT AND THE TOTAL AREA OF TISSUE IN THE UPPER AND LOWER HALVES OF 30 BREASTS

		Upper Half		Lower Half	
Side	Age	NFC	Total Area	NFC	Total Area
L	19	490.22	1325.98	406.20	686.31
R	19	144.73	533.96	180.44	503.83
R*	21	634.77	990.42	357.14	529.28
L*	21	412.69	849.29	276.98	385.33
R*	22	171.20	1014.14	219.45	1295.94
L*	22	315.57	1374.04	187.91	937.82
L	25	447.12	884.89	229.52	641.89
R*	25	524.09	751.65	220.72	311.82
L*	25	201.75	358.73	226.0	346.79
R	29	549.44	1070.27	357.75	438.05
R	30	112.43	537.47	91.04	320.71
L	35	773.10	1392.82	437.73	989.68
R	37	889.36	1368.51	296.96	685.15
R	38	324.29	2083.23	146.18	887.51
R	40	171.79	411.88	271.06	381.10
R	41	381.80	1680.76	430.14	1027.25
L	41	210.10	382.95	189.09	396.59
R	42	365.85	858.33	321.42	723.80
L	44	218.10	664.60	173.62	623.91
L	46	132.20	362.35	247.39	383.48
L	46	355.19	777.76	87.18	523.79
L	46	798.23	2746.49	269.15	924.03
L	53	238.24	606.41	125.61	279.81
L	55	137.07	664.20	71.79	259.47
R*	56	358.40	487.69	228.09	313.25
L*	56	426.25	513.05	324.60	469.23
L	57	89.14	1152.51	95.55	761.82
R*	63	77.06	572.39	21.43	300.40
L*	63	95.59	503.58	88.83	263.18
L	73	267.16	1331.15	102.84	516.41

R: Right L: Left * Pairs of Right and Left Breasts

TABLE 29:

AREA IN CM^2 OF THE NON-FATTY COMPONENT AND THE TOTAL AREA OF TISSUE RECORDED FOR THE UOQ + LIQs AND THE UIQ + LOQs IN 30 BREASTS

		NFC		Total Area	
Side	Age	UOQ + LIQ	UIQ + LOQ	UOQ + LIQ	UIQ + LOQ
L	19	462.78	433.64	1050.84	961.45
R	19	179.67	145.50	524.05	513.74
R*	21	608.80	383.11	878.73	640.97
L*	21	374.19	315.48	653.18	581.44
R*	22	200.87	189.78	1224.45	1085.63
L*	22	244.63	258.85	1292.24	1019.62
L	25	358.73	317.91	765.72	761.06
R*	25	447.54	297.27	632.04	431.43
L*	25	208.77	218.98	351.68	353.84
R	29	600.56	306.63	811.52	696.80
R	30	104.29	99.18	389.19	468.99
L	35	718.41	492.42	1311.22	1071.28
R	37	682.70	503.62	1151.77	901.89
R	38	221.00	249.47	1613.52	1357.22
R	40	244.34	198.51	418.40	374.58
R	41	334.88	477.06	1233.12	1474.89
L	41	272.73	126.46	469.06	310.48
R	42	337.45	349.82	741.66	840.47
L	44	165.39	226.33	610.82	677.69
L	46	210.94	168.65	461.73	284.10
L	46	256.75	185.62	456.91	844.64
L	46	577.93	516.45	1977.94	1692.58
L	53	184.67	179.18	496.05	390.17
L	55	100.78	108.08	461.44	462.23
R*	56	323.71	262.78	432.76	368.18
L*	56	376.13	374.72	490.99	491.29
L	57	94.66	90.03	965.69	948.64
R*	63	40.14	58.35	410.17	462.62
L*	63	96.83	87.59	509.63	257.13
L	73	179.91	190.09	936.81	910.75

R: Right L: Left *Pairs of Right and Left Breasts.

TABLE 30:

AREA IN CM^2 OF THE NON-FATTY COMPONENT IN THE SUPERFICIAL, MIDDLE AND DEEP THIRDS OF 29 BREASTS

Side	Age	Superficial Third	Middle Third	Deep Third
L	19	271.66	413.61	211.15
R	19	116.16	126.42	82.59
R	21	338.07	392.24	261.60
R*	22	118.99	196.39	75.27
L*	22	116.91	276.87	109.70
L	25	80.80	295.33	300.51
R*	25	246.10	253.21	245.50
L*	25	73.83	235.70	118.22
R	29	148.94	385.29	372.96
R	30	4.52	82.06	116.89
L	35	65.09	456.72	689.02
R	37	168.35	508.40	509.57
R	38	44.29	128.32	297.86
R	40	37.93	192.05	212.87
R	41	151.66	365.72	294.56
L	41	29.38	165.33	204.48
R	42	108.56	272.86	305.85
L	44	15.36	141.87	234.49
L	46	127.76	145.98	105.85
L	46	130.60	206.63	105.14
L	46	160.51	552.08	381.79
L	53	22.21	106.57	235.07
L	55	13.14	106.89	88.83
R*	56	183.88	247.43	155.18
L*	56	226.74	308.33	215.78
L	57	29.41	72.26	83.02
R*	63	1.97	60.49	36.03
L*	63	10.71	71.29	102.42
L	73	20.63	276.10	73.27

R: Right L: Left * Pairs of Right and Left Breasts

TABLE 31:

STATISTICAL RESULTS FOLLOWING THE APPLICATION OF WILCOXON SUM RANK TESTS TO THE RESULTS FOR THE NON-FATTY COMPONENT AND THE TOTAL AREA DERIVED FROM TABLES 24-25, 27-29.

Lobule scores	NFC				Total Area			
	30 breasts	p	25 breasts	p	30 breasts	p	25 breasts	p
UOQ > UIQ	23	< 0.01	20	< 0.01	23	< 0.01	19	< 0.01
LOQ > LIQ	21	< 0.05	18	< 0.05	21	not sig.	17	not sig.
UIQ > LOQ	19	not sig.	16	not sig.	25	< 0.01	22	< 0.01
Outer half > inner half	23	< 0.05	19	< 0.05	20	< 0.01	17	< 0.01
Upper half > lower half	22	< 0.01	18	< 0.01	27	< 0.01	22	< 0.01
UOQ + LIQ > UIQ + LOQ	21	< 0.05	19	< 0.05	22	< 0.01	20	< 0.01

TABLE 32:

PERCENTAGE INCIDENCE OF LOBULE TYPES IN 47 WHOLE BREASTS

Side	Age (in yrs)	Feather	Intermediate	Solid	Microcystic
R*	19	0	53.0	41.7	5.3
L*	19	0	38.6	56.3	5.1
R	19	1.9	27.7	65.3	4.9
R*	21	0	68.0	32.0	0
L*	21	2.9	51.2	45.9	0
L	22	18.8	18.3	62.9	0
R*	25	0	9.3	90.7	0
L*	25	0	10.0	90.0	0
L	25	0.2	23.8	76.0	0
L	26	0	44.3	55.3	0.4
R	29	1.9	13.8	82.8	1.5
R	29	4.0	15.0	81.0	0
R	30	0	17.8	82.2	0
L	31	13.2	32.3	54.2	0.3
R	34	0.8	57.4	40.4	1.4
L	35	0	69.5	25.3	4.2
R	37	0.2	5.7	91.6	2.5
L	38	1.2	29.6	62.7	6.5

TABLE 32 (contd.)

Side	Age (in yrs)	Feather	Intermediate	Solid	Microcystic
R	38	58.7	41.7	3.3	3.0
L	39	2.1	20.0	71.4	6.4
R	40	8.8	54.3	34.4	2.5
L	41	5.3	55.3	34.1	5.3
R	41	1.1	51.2	21.6	26.1
R	42	0	42.5	24.1	33.4
L	43	7.9	35.0	51.3	5.8
L	43	7.1	15.8	76.7	0.4
R*	44	13.8	56.4	23.3	6.5
L*	44	24.0	56.4	15.8	3.8
R	46	0	61.2	31.2	7.6
L	46	86.7	13.3	0	0
L	46	1.3	87.1	6.6	5.0
L	46	1.1	50.7	40.2	8.0
R*	49	11.1	20.6	63.9	4.4
L*	49	8.9	22.8	64.4	3.9
R*	50	3.1	78.1	6.3	12.5
L*	50	17.2	60.8	5.6	16.4
L	50	17.2	41.4	34.2	7.2
L	50	3.4	83.4	8.5	4.7

TABLE 32 (contd.)

Side	Age (in yrs)	Feather	Intermediate	Solid	Microcystic
L	50	0.5	65.5	34.0	0
L	53	0	74.4	20.6	5.0
L	54	41.9	35.0	15.0	8.1
L	55	5.6	94.4	0	0
R*	56	0.5	65.5	25.6	9.6
L*	56	0	68.8	28.7	2.5
L	57	65.9	33.4	0.5	0.2
R	63	33.9	50.6	1.1	14.4
L	73	5.0	38.9	51.5	4.6

R - Right 1 - Left *Matching pairs of right and left breasts

TABLE 33:

INCIDENCE OF CYSTS, APOCRINE CYSTS AND FIBROADENOMATA IN 47 WHOLE BREASTS

Side	Age (in yrs)	F	c	cc	Ac	cAc	cc & Ac
R*	19	1	-	-	1	1	1
L*	19	-	-	1	-	-	-
R	19	-	-	-	-	-	-
R*	21	-	-	-	-	-	-
L*	21	-	-	-	-	-	-
L	22	-	-	-	-	-	-
R*	25	-	-	-	-	-	-
L*	25	-	-	-	-	-	-
L	25	-	1	6	-	-	-
L	26	-	4	3	5	-	-
R	29	3	3	4	-	1	-
R	29	-	-	-	-	1	-
R	30	-	-	-	-	-	-
L	31	-	-	2	-	14	-
R	34	3	1	2	2	2	-
L	35	-	-	-	-	-	-
R	37	1	-	8	-	-	-
L	38	1	10	-	11	-	-
R	38	-	-	-	5	-	-
L	39	-	10	-	3	-	-
R	40	-	1	-	-	4	1
L	41	-	6	25	3	5	1
R	41	-	30	4	1	2	-
R	42	-	37	5	-	4	2
L	43	-	-	-	-	-	-
L	43	-	1	8	1	3	-
R*	44	3	11	43	10	13	-
L*	44	-	13	10	4	3	-
R	46	-	30	19	-	6	1
L	46	-	-	-	-	-	-
L	46	-	3	-	1	-	-
L	46	-	20	50	2	16	10
R*	49	1	6	5	2	3	-
L*	49	-	5	15	2	2	-

TABLE 33 (contd.)

Side	Age (in yrs)	F	c	cc	Ac	cAc	cc & Ac
R*	50	-	8	-	1	-	-
L*	50	-	7	4	-	2	-
L	50	-	19	34	2	2	29
L	50	-	4	5	1	2	-
L	50	-	-	-	-	-	-
L	53	-	5	9	-	3	22
L	54	-	1	6	-	1	-
L	55	-	-	-	-	-	-
R*	56	-	7	3	-	11	-
L*	56	3	14	14	1	-	12
L	57	-	-	-	-	-	-
R	63	-	-	8	-	-	-
L	73	2	3	3	3	23	15

F - fibroadenomata

c - individual cysts

cc - groups of cysts

Ac - individual apocrine cysts

cAc - groups of apocrine cysts

cc & Ac - groups of cysts and apocrine cysts

TABLE 34: DETAILS OF BREAST BIOPSIES

Day of cycle	Age (in yrs)	Side	Parity	Pill	No. of blocks examined	Diagnosis of the biopsy
1	23	R & L	0 + 0	+	6	Benign mammary dysplasia
1	30	L	0 + 0	-	2	Benign mammary dysplasia
1	39	R	2 + 0	+	1	Fibrosis and atrophy
1	25	L	2 + 0	+	1	Normal breast tissue showing secretory activity
2	21	L	1 + 0	+	2	Benign fibrocystic disease with papillomatosis
2	23	L	1 + 1	-	1	Fibroadenoma
3	44	L	3 + 1	+	2	Benign mammary dysplasia with benign cyst
3	39	L	0 + 0	-	1	Benign mammary dysplasia
4	28	L	1 + 0	+	1	Mild benign mammary dysplasia
5	39	L	0 + 0	-	1	Benign mammary dysplasia
5	42	L	2 + 1	-	2	Fibroadenoma
5	19	R	0 + 0	-	1	Normal breast tissue
6	32	R & L	1 + 0	-	4	Mild benign mammary dysplasia
6	36	L	3 + 1	-	2	Fibroadenomata
7	34	L	2 + 0	-	2	Sclerosing adenosis
9	39	R	3 + 0	-	1	Benign mammary dysplasia, fibrocystic areas
10	36	L	0 + 0	+	2	Fibroadenoma and mild benign mammary dysplasia
10	45	R	2 + 0	-	1	Benign mammary dysplasia and apocrine cysts
11	22	L	0 + 0	-	1	Normal breast with some increase in fibrous tissue
12	39	R	0 + 0	-	1	Benign mammary dysplasia
12	53	L	0 + 0	-	1	Benign mammary dysplasia
13	23	R	0 + 0	-	2	Fibroadenomata
13	29	R		+	1	Mild chronic inflammatory cell infiltrate
15	32	L	0 + 0	-	1	Benign mammary dysplasia
16	24	R	0 + 0	+	2	Normal breast tissue
17	38	R	0 + 0	+	1	Fibroadenoma
17	38	R	0 + 0	-	1	Fibroadenoma
17	40	R	3 + 0	-	1	Fibroadenoma
19	19	R	0 + 0	-	1	Fibrosis and small fibroadenomatous areas
20	27	R	2 + 0	-	2	Benign mammary dysplasia
21	20	L	0 + 0	-	1	Normal breast tissue
21	21	L	0 + 0	-	2	Fibroadenoma
22	34	R	0 + 0	-	2	Benign mammary dysplasia
23	19	R	0 + 0	+	1	Fibroadenoma
23	32	R	3 + 1	-	2	Normal breast tissue
23	23	R	0 + 0	+	1	Periductular fibrosis
24	24	R	0 + 1	-	1	Fibroadenoma
25	19	R	0 + 0	-	1	Mild fibrosis
26	19	R	0 + 0	-	3	Pericanalicular fibroadenoma
26	27	R	2 + 2	-	1	Benign area of active fibrosis
26	26	R	1 + 0	-	2	Non specific chronic mastitis
27	39	L	6 + 0	-	3	Benign mammary dysplasia with sclerosing lobules and some calcification
28	28	L	0 + 0	-	2	Normal breast tissue with some increase in fibrous tissue

Total: 43 biopsies
70 blocks examined

R: right
L: left
+ currently on pill
- not currently on pill

+ - currently on pill
- - not currently on

TABLE 35:

PROFORMA FOR THE ASSESSMENT OF MUCOPOLYSACCHARIDE STAINING REACTIONS IN THE BREAST

BIOPSY NO:	SECTION NO:			DATE:		
	+	++	+++	R	R/B	N
Lobule No: Number of Ductules						
Secretions						
Granules						
Stroma						
Lobule No: Number of Ductules						
Secretions						
Granules						
Stroma						
Lobule No: Number of Ductules						
Secretions						
Granules						
Stroma						
Lobule No: Number of Ductules						
Secretions						
Granules						
Stroma						

TABLE 36:

CASE DATA FOR THE STUDY OF MUCOPOLYSACCHARIDE STAINING REACTIONS IN NORMAL TISSUE FROM 43 BREAST BIOPSIES

Case No.	Block No.	Age (in yrs)	Side	DOC	No. of lobules	No. of ductules	Pill	Parity
1	1	23	R	1	43	1320	+	N
	2		R		66	2138		
	3		R		13	134		
	4		L		12	285		
	5		L		19	719		
	6		L		19	758		
2	1	30	L	1	29	1250	-	P
	2		L		25	890		
3	1	39	R	1	26	796	+	P
4	1	25	L	1	46	1432	+	P
5	1	21	L	2	15	209	+	P
	2		L		34	538		
6	1	23	L	2	43	1275	-	P
7	1	44	L	3	35	521	+	P
	2		L		18	211		
8	1	39	L	3	38	1299	-	N
9	1	19	L	3	64	1475	-	N
10	1	28	L	4	31	707	+	P
11	1	39	L	5	58	1339	-	N
12	1	42	L	5	31	703	-	P
	2		L		35	942	-	P
13	1	19	R	5	53	1089	-	N
14	1	32	L	6	42	1414	-	P
	2		R		57	1563		
	3		R		49	1145		
	4		R		43	961		
15	1	36	L	6	66	1850	-	P
	2		L		38	828		

TABLE 36 (contd.)

Case No.	Block No.	Age (in yrs)	Side	DOC	No. of lobules	No. of ductules	Pill	Parity
16	1	34	L	7	42	1020	-	P
	2		L		26	556		
17	1	39	R	9	26	754	-	P
18	1	36	L	10	33	539	+	N
	2		L		20	313		
19	1	45	R	10	34	983	-	P
20	1	22	L	11	14	111	-	N
21	1	39	R	12	47	1424	-	N
22	1	53	L	12	29	414	-	N
23	1	23	R	13	51	2210	-	N
	2				54	3123	-	N
24	1	29	R	13	44	912	+	P
25	1	32	L	15	72	2208	-	N
26	1	24	R	16	60	1808	+	N
	2		R		28	911	+	N
27	1	24	R	17	42	600	+	N
28	1	38	R	17	35	605	-	N
29	1	40	R	17	44	1865	-	P
30	1	27	R	20	57	2197	-	P
	2		R		24	540		
31	1	20	L	21	22	293	-	N
32	1	21	L	21	26	888	-	N
	2		L		42	1804		
33	1	34	R	22	16	399	-	N
	2		R		36	887		
34	1	19	R	23	53	1539	+	N
35	1	32	R	23	67	3152	-	P
	2		R		42	1113		
36	1	23	R	23	14	225	+	N
37	1	24	R	24	11	136	-	N
38	1	19	R	25	78	1172	-	N

TABLE 36 (contd.)

Case No.	Block No.	Age (in yrs)	Side	DOC	No. of lobules	No. of ductules	Pill	Parity
39	1	19	R	26	26	387	-	N
	2		R		56	1320		
	3		R		19	500		
40	1	27	R	26	64	1461	-	P
41	1	26	R	26	26	644	-	P
	2		R		5	538		
42	1	39	L	27	40	740	-	P
	2		L		31	1168		
	3		L		42	900		
43	1	28	L	28	12	120	-	N
	2		L		59	1457		

DOC: day of cycle

\+ : on pill

\- : not on pill

Parity - N: nulliparous

P: parous

TABLE 37:

ASSESSMENT OF MUCOPOLYSACCHARIDE STAINING REACTIONS IN LOBULES OF NORMAL TISSUE FROM 43 BREAST BIOPSIES

Case No.	Block No.	DOC	Stroma			Granules			Secretions			R	R/B	N
			+	++	+++	+	++	+++	+	++	+++			
1	1	1	18	7	0	13	19	10	5	21	17	22	21	18
	2		19	33	0	25	9	15	28	22	16	33	30	37
	3		3	8	0	2	6	5	1	3	9	8	10	10
	4		6	5	0	4	4	4	8	3	1	8	5	2
	5		7	0	0	10	4	0	4	4	10	12	8	7
	6		18	0	0	10	4	1	5	6	8	18	8	5
2	1	1	9	0	0	1	11	16	14	2	0	16	0	0
	2		20	0	0	5	3	16	10	3	5	17	2	1
3	1	1	14	7	0	4	7	15	2	5	18	9	15	9
4	1	1	22	17	0	22	4	7	18	11	6	13	11	21
5	1	2	0	0	0	6	2	1	6	2	7	9	4	6
	2		6	0	0	6	3	17	9	4	20	20	12	22
6	1	2	5	0	0	8	20	15	22	12	9	35	37	7
7	1	3	25	6	0	2	5	27	15	10	8	19	26	2
	2		6	6	5	4	2	12	7	7	4	1	13	6
8	1	3	14	6	0	9	11	17	13	13	9	33	19	20
9	1	3	32	29	2	1	13	50	26	6	23	45	21	9

TABLE 37 (contd.)

Case No.	Block No.	DOC	Stroma			Granules			Secretions			R	R/B	N
			+	++	+++	+	++	+++	+	++	+++			
10	1	4	0	0	0	4	10	17	7	12	12	28	27	4
11	1	5	16	1	0	13	20	17	13	11	31	32	46	8
12	1	5	3	1	0	1	0	0	24	3	1	16	9	6
	2		11	7	1	16	5	2	16	9	4	24	16	8
13	1	5	19	4	0	0	17	36	10	17	23	52	2	32
14	1	6	2	1	0	14	9	16	7	16	17	38	17	0
	2		14	2	0	14	17	24	14	19	23	51	36	16
	3		0	0	0	24	11	2	6	10	31	47	37	0
	4		10	23	3	17	4	18	18	16	7	33	8	8
15	1	6	24	0	0	29	26	10	18	18	27	44	36	28
	2		12	25	0	12	13	12	13	14	11	14	15	22
16	1	7	17	19	0	2	8	32	5	8	28	25	36	21
	2		12	0	0	7	7	11	7	8	11	15	20	17
17	1	9	6	3	0	7	8	8	15	7	2	10	14	7
18	1	10	15	16	1	2	7	21	1	5	27	20	14	25
	2		12	7	0	0	0	20	4	2	14	11	6	18
19	1	10	5	0	0	4	13	17	0	6	28	31	19	6
20	1	11	5	0	0	0	0	14	3	1	10	13	2	4

TABLE 37 (contd.)

Case No.	Block No.	DOC	Stroma			Granules			Secretions			R	R/B	N
			+	++	+++	+	++	+++	+	++	+++			
21	1	12	7	0	0	25	3	0	24	17	6	42	4	10
22	1	12	16	0	0	2	5	19	1	5	21	15	14	13
23	1	13	5	0	0	6	2	2	19	18	11	26	25	19
	2		26	4	0	26	17	5	33	9	3	43	3	28
24	1	13	1	0	0	22	7	9	9	9	25	36	10	8
25	1	15	33	9	0	27	25	19	26	22	18	67	13	13
26	1	16	48	9	0	22	22	13	21	13	25	35	33	25
	2		17	5	3	5	7	16	9	3	14	15	10	16
27	1	17	10	7	9	10	4	24	6	6	26	38	2	12
28	1	17	5	0	0	2	6	27	13	8	9	16	20	4
29	1	17	1	0	0	10	6	3	22	12	7	31	17	2
30	1	20	1	0	0	28	3	0	7	11	39	55	43	17
	2		0	0	0	15	5	0	3	7	12	20	12	6
31	1	21	6	7	4	3	4	2	13	2	4	19	0	0
32	1	21	19	2	0	10	7	8	9	7	10	22	22	2
	2		25	14	0	16	13	8	12	13	14	28	27	4
33	1	22	1	0	0	2	0	0	10	6	0	16	1	0
	2		0	0	0	6	0	0	22	7	2	25	10	4

TABLE 37 (contd.)

Case No.	Block No.	DOC	Stroma			Granules			Secretions			R	R/B	N
			+	++	+++	+	++	+++	+	++	+++			
34	1	23	8	14	23	0	6	46	0	2	50	51	23	34
35	1	23	9	42	2	0	7	60	5	16	44	65	18	8
	2		4	18	0	6	6	24	7	12	23	42	14	0
36	1	23	7	1	0	0	0	14	4	3	7	8	10	4
37	1	24	4	3	0	0	1	10	6	0	4	8	3	3
38	1	25	42	25	3	19	36	19	20	16	31	60	15	13
39	1	26	7	8	0	2	2	22	1	3	22	24	13	8
	2		35	0	0	11	25	17	2	6	47	55	37	15
	3		7	0	0	2	8	9	1	7	11	19	6	15
40	1	26	10	0	0	10	21	31	10	19	34	43	31	10
41	1	27	3	10	12	2	14	10	10	6	8	22	4	6
	2		0	0	4	0	0	5	1	1	0	1	1	0
42	1	27	18	4	10	4	6	29	18	13	5	15	22	3
	2		12	5	2	5	5	21	15	9	5	7	24	6
	3		2	1	0	2	0	0	20	8	10	24	27	8
43	1	28	7	2	0	4	0	1	2	4	5	2	8	5
	2		24	7	0	13	4	0	21	21	16	46	18	5

DOC: Day of menstrual cycle
R: PAS staining
R/B: PAS/AB staining (predominantly PAS)
N: PAS/AB staining (predominantly AB)

Staining reactions: + mild
++ moderate
+++ heavy

TABLE 38:

SCORES FOR MUCOPOLYSACCHARIDE STAINING REACTIONS IN BREAST LOBULES

Case No.	STR score	GRA score	SEC score	R	N	R/B
1	0.744	1.884	2.279	0.512	0.419	0.488
2	0.310	2.448	0.621	0.552	0	0
3	1.077	2.423	2.538	0.346	0.346	0.577
4	1.217	1.109	1.261	0.283	0.457	0.239
5	0	0.867	2.067	0.600	0.400	0.267
6	0.116	2.163	1.698	0.814	0.163	0.860
7	1.057	2.657	1.686	0.543	0.057	0.743
8	0.684	2.158	1.737	0.868	0.526	0.500
9	1.500	2.766	1.672	0.703	0.141	0.328
10	0	2.419	2.161	0.903	0.129	0.871
11	0.310	1.793	2.207	0.552	0.138	0.793
12	0.161	0.032	1.065	0.516	0.194	0.290
13	0.509	2.679	2.132	0.943	0.604	0.037
14	0.095	1.905	2.143	0.905	0	0.405
15	0.364	1.682	1.045	0.667	0.424	0.545
16	1.310	2.714	2.500	0.595	0.500	0.857
17	0.462	1.808	1.346	0.385	0.269	0.538
18	1.515	2.394	2.788	0.606	0.758	0.424
19	0.147	2.382	2.824	0.912	0.176	0.559
20	0.357	3	2.500	0.929	0.286	0.143
21	0.149	0.660	1.617	0.894	0.213	0.085
22	0.552	2.379	2.552	0.517	0.448	0.483
23	0.098	0.314	1.725	0.510	0.373	0.490
24	0.022	1.432	2.318	0.818	0.182	0.227
25	0.708	1.861	1.722	0.931	0.181	0.181
26	1.100	1.750	2.033	0.583	0.417	0.550
27	1.214	2.143	2.286	0.905	0.286	0.047
28	0.143	2.714	1.600	0.457	0.114	0.571
29	0.022	0.705	1.523	0.705	0.045	0.386
30	0.017	0.596	2.561	0.965	0.298	0.754

TABLE 38 (Contd.)

Case No.	STR score	GRA score	SEC score	R	N	R/B
31	1.455	0.773	1.318	0.864	0	0
32	0.885	1.846	2.038	0.846	0.076	0.846
33	0.0625	0.125	1.375	1.000	0	0.062
34	1.981	2.830	2.906	0.962	0.642	0.434
35	1.478	2.896	2.522	0.970	0.119	0.269
36	0.643	3.000	2.214	0.571	0.286	0.714
37	0.909	2.909	1.636	0.727	0.273	0.273
38	1.295	1.897	1.859	0.769	0.167	0.192
39	0.885	2.769	2.808	0.923	0.308	0.500
40	0.156	2.266	2.344	0.672	0.156	0.484
41	2.269	2.308	1.769	0.846	0.231	0.154
42	1.400	2.575	1.475	0.375	0.075	0.550
43	0.917	0.583	2.083	0.167	0.417	0.667

STR score: stromal score

GRA score: intracellular granule score

SEC score: secretion score

R: PAS staining only

N: equivalent PAS and AB staining intensity

R/B: predominantly PAS staining

TABLE 39:

REPRODUCIBILITY OF THE ASSESSMENT OF ALCIAN BLUE STAINING IN THE INTRALOBULAR STROMA OF LOBULES FROM 25 BREAST BIOPSIES SELECTED AT RANDOM FROM THE EXPERIMENTAL SERIES OF 43 CASES

Case No.	Block No.	No. of lobules	Stroma			STR score
			+	++	+++	
1	2	66	18	26	0	1.288
1	4	9	1	8	0	1.333
3	1	26	15	2	0	1.077
7	1	34	18	1	0	1.057
7	2	17	8	4	5	1.833
12	2	33	18	6	0	0.800
15	1	65	33	0	0	0.364
16	2	24	18	0	0	0.462
17	1	39	12	0	0	0.462
18	1	35	21	15	1	1.515
19	1	34	3	0	0	0.147
22	1	28	7	0	0	0.552
23	1	51	3	0	0	0.980
25	1	74	41	19	2	0.708
27	1	40	4	7	8	0.214
30	1	57	2	0	0	0.017
31	1	22	9	9	3	1.455
32	1	26	19	3	0	0.885
33	1	15	1	0	0	0.062
34	1	52	10	18	12	1.981
35	2	42	3	20	0	0.952
39	1	25	15	2	0	0.885
40	1	63	4	0	0	0.156
41	1	25	4	13	8	2.269
43	1	12	8	3	0	0.917

TABLE 40:

RESULTS FOR THE ASSESSMENTS OF INTERCELLULAR GRANULES WITHIN DUCTULAR CELLS OF LOBULES OF NORMAL TISSUE FROM 43 BREAST BIOPSIES

Case No.	Block No.	DOC	No. of lobules	Subluminal Granules			Not Subluminal	
				LG	SG	SG + LG	L	O
1	1	1	48	26	7	1	37	14
	2		50	21	6	2	24	21
	3		11	1	5	0	3	5
	4		15	0	4	3	5	8
	5		20	1	12	3	9	4
	6		24	10	5	7	20	2
2	1	1	22	7	0	1	5	14
	2		28	5	0	0	6	23
3	1	1	26	4	6	0	5	16
4	1	1	49	2	31	3	10	13
5	1	2	17	3	2	0	5	12
	2		34	0	11	8	25	15
6	1	2	42	3	11	0	10	28
7	1	3	30	13	2	0	16	15
	2		15	1	7	4	4	3
8	1	3	34	17	2	6	12	9
9	1	3	50	7	17	1	10	25
10	1	4	37	1	10	3	3	23
11	1	5	50	6	16	4	10	24
12	1	5	36	0	3	0	4	33
	2		32	0	3	6	3	29
13	1	5	50	0	7	0	7	43
14	1	6	41	16	8	2	9	15
	2		50	3	14	2	8	31
	3		45	3	6	1	6	35
	4		46	5	12	1	10	28

TABLE 40 (contd.)

Case No.	Block No.	DOC	No. of lobules	Subluminal Granules			Not Subluminal	
				LG	SG	SG + LG	L	O
15	1	6	50	6	1	0	14	43
	2	6	34	0	9	0	10	25
16	1	7	40	0	31	6	14	3
	2		30	1	13	2	6	14
17	1	9	26	0	8	2	10	16
18	1	10	31	1	21	3	12	6
	2		23	0	18	5	12	0
19	1	10	39	25	4	0	32	10
20	1	11	14	2	5	1	11	6
21	1	12	42	5	1	0	12	36
22	1	12	32	6	9	5	11	12
23	1	13	50	2	9	0	10	39
	2		49	2	9	0	0	38
24	1	13	40	2	1	0	11	37
25	1	15	50	6	5	2	16	37
26	1	16	50	5	7	1	4	37
	2		31	7	5	0	9	19
27	1	17	37	5	2	1	15	29
28	1	17	32	0	30	1	4	1
29	1	17	41	0	10	1	5	30
30	1	20	50	3	1	2	16	44
	2		25	3	2	0	0	20
31	1	21	20	0	6	0	2	14
32	1	21	27	0	3	0	5	24
	2		43	5	9	0	4	27
33	1	22	15	3	1	0	3	11
	2		40	6	12	3	13	19
34	1	23	50	40	0	0	50	10
35	1	23	50	10	5	8	33	27
	2		50	15	2	0	30	33

TABLE 40 (contd.)

Case No.	Block No.	DOC	No. of lobules	Subluminal Granules			Not Subluminal	
				LG	SG	SG + LG	L	O
36	2	23	50	16	6	0	18	28
37	1	24	12	1	8	0	2	3
38	1	25	50	13	2	0	28	35
39	1	26	30	9	3	0	12	18
	2		50	12	4	0	8	34
	3		18	5	0	0	7	13
40	1	26	50	16	6	0	18	28
41	1	26	29	10	3	1	21	15
	2		5	1	0	0	0	4
42	1	27	39	1	24	4	8	10
	2		32	3	12	2	5	15
	3		45	1	19	0	4	25
43	1	28	13	1	4	8	7	27
	2		50	6	9	0	5	16

DOC: day of menstrual cycle

LG: large granules

SG: small granules

L: large granules not in a subluminal position within the cell

O: lobules with no intracellular granules

TABLE 41:

ASSESSMENT OF ALCIAN BLUE/PERIODIC ACID SCHIFF STAINING FOLLOWING DIASTASE DIGESTION OF INTRACELLULAR GRANULES WITHIN DUCTULAR CELLS OF LOBULES FROM 16 BREAST BIOPSIES

Case No.	Block No.	No. of lobules	LG	SG	SG + LG	L	O
1	2	50	29	0	1	10	20
	4	14	3	4	3	1	2
4	1	50	20	0	0	15	30
5	1	15	7	2	0	1	6
7	1	30	27	2	3	18	3
12	1	35	2	0	0	5	28
	2	32	4	1	0	7	25
15	1	50	19	5	6	18	20
18	1	31	6	13	9	3	6
20	1	14	4	1	1	3	8
23	2	50	15	12	3	15	20
26	1	50	25	6	6	22	13
	2	29	24	0	0	21	15
32	1	27	8	4	5	6	10
33	1	15	5	1	0	6	7
35	1	50	30	2	0	20	18
37	1	11	4	3	1	7	3
39	2	50	27	4	5	26	14
42	1	40	7	6	1	14	27
	3	45	6	3	0	12	30

LG: Large granules
SG: Small granules
L: Large granules not in a subluminal position within the cell
O: Lobules with no intracellular granules

Reprinted from the Journal of Clinical Pathology
Volume 34, pages 1189-1191, 1981.

An automated technique for the rapid processing of breast tissue for subgross examination

SARAH L MANTON, DJP FERGUSON, TJ ANDERSON

British Medical Association, Tavistock Square, London WC1H 9JR

An automated technique for the rapid processing of breast tissue for subgross examination

SARAH L MANTON, DJP FERGUSON, TJ ANDERSON
Department of Pathology, University of Edinburgh Medical School, Teviot Place, Edinburgh

In the study of normal and abnormal breast tissue, additional three dimensional information on the parenchyma can be obtained from the subgross examination of breast biopsies. In this paper an automated technique is described for the rapid processing of breast biopsies for subgross examination. It is adapted from methods previously described for subgross examination of whole breasts.[1 2]

Material and methods

The breast biopsies were fixed in 4% formaldehyde in phosphate buffer[3] for a minimum of 6 h. The tissue for subgross examination was cut into approximately 1-2 mm thick slices using a skin graft knife blade.

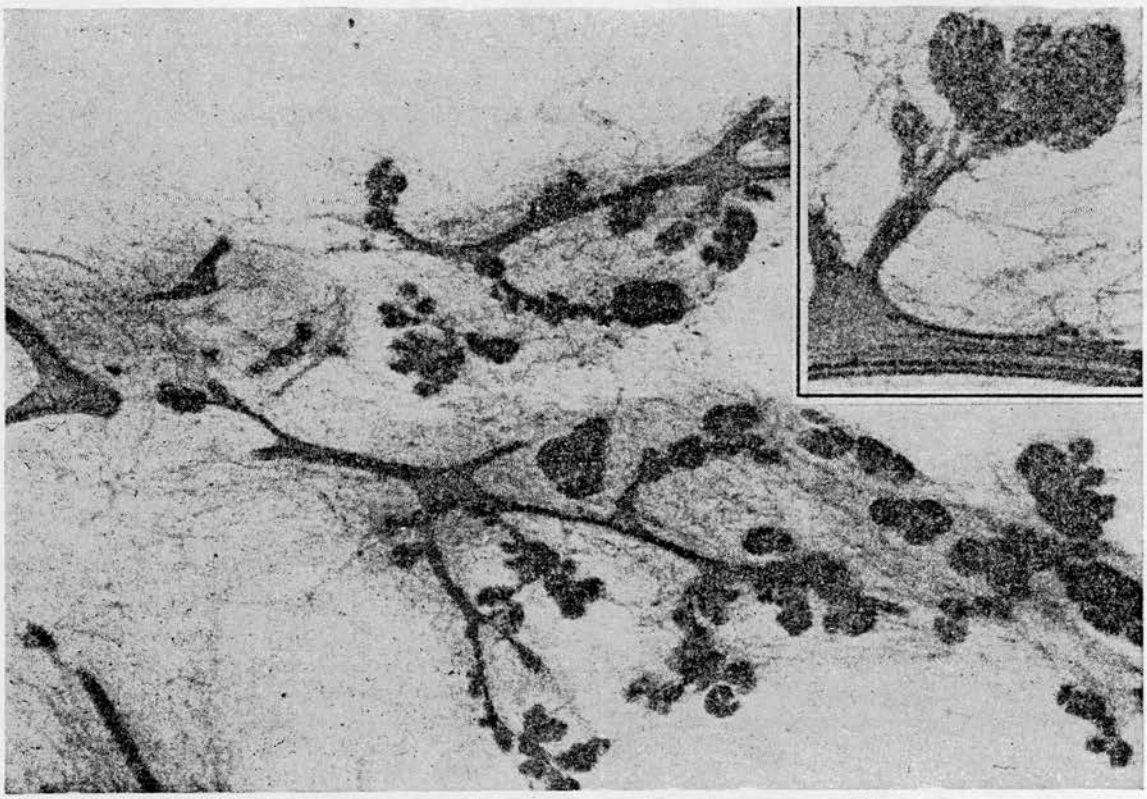
The slices were placed in wire mesh histokinette baskets and processed in a histokinette using the following schedule:

1 Wash in running water 15 min.
2 Stain with Delafield's haematoxylin 1 h.
3 Wash in running water 1 h.
4 Decolourise in 2% acid alcohol (hydrochloric acid/ethanol) 1 h.
5 Differentiate by washing in running water 30 min.
6 Dehydrate in 95% ethanol 3 h.
7 Dehydrate in 99% ethanol 3 h.
8 Dehydrate in 99% ethanol 3 h.
9 Dehydrate in 100% ethanol 4 h.
10 Dehydrate in 100% ethanol 4 h.
11 Clear in methyl salicylate 2 h.

To allow washing in running water, the histokinette beakers were adapted with an inlet and outlet which were connected to the water system.

After clearing, the slices were removed from the histokinette and placed in Kapak/Scotchpak heat-sealable pouches. Excess methyl salicylate and air were expelled prior to heat sealing the pouches. The tissue slices can now be examined with a dissecting microscope. Areas of interest can be readily identified and excised for histological examination.

Accepted for publication 27 April 1981



The subgross appearance of a tissue slice containing normal parenchymal structures. × 10. (Insert: Detail of the ducts and lobules × 20.)

Results and discussion

The times followed in this schedule gave optimal results in our laboratory. However, they should only be used as a guide and may have to be varied depending, for example, on the pH of the tap water. We have found that the staining time is not critical nor is the decolourisation in acid alcohol. The critical step in obtaining optimum results is the wash subsequent to the acid alcohol and this is the stage which may require variation depending on the reaction of the tissue sample. After acid alcohol the tissue appears pink and as washing proceeds the parenchyma becomes blue in colour. The point at which to stop washing and begin dehydration is when the lobules and ducts are a deep blue colour and the background stroma appears white or has a slight bluish tint.

The samples to be studied histologically after subgross examination are returned to absolute alcohol and processed for embedding in either wax or plastic. We have found that this technique does not affect subsequent histological staining. Haematoxylin and eosin, alcian blue/periodic acid-Schiff and Feulgen stains have been successfully carried out on the tissue.

The value of the subgross technique is the appreciation that it gives of the three dimensional appearance of the ducts and lobules, including the duct pattern, lobular distribution, and lobular architecture (Figure 1). Furthermore, it allows examination of relatively large tissue samples to identify small atypical areas or regions of altered architecture which can subsequently be removed for histological examination. By applying the criteria described by Wellings *et al.*[2] it is possible to identify certain types of lesions at the subgross level.

The main advantages of this subgross technique over those described previously[1 2] are that it is fully automated, only requires 24 h to complete and a number of samples can be processed simultaneously.

The technical assistance of Mrs M McHenry and Miss L Dunlop is gratefully acknowledged. This work was supported by a grant from the Scottish Home and Health Department to TJA.

References

[1] Ingleby H, Holly C. A method for the preparation of serial slices of the breast. *Bull Int Assoc Med Museums* 1939;**19**:93-6.

[2] Wellings SR, Jensen HM, Marcum RG. An atlas of subgross pathology of the human breast with special reference to possible precancerous lesions. *J Natl Cancer Inst* 1975;**55**:231-73.

[3] Carson FL, Martin JH, Lynn JA. Formalin fixation for electron microscopy. A re-evaluation. *Am J Clin Pathol* 1973;**59**:365-73.

Requests for reprints to: Dr TJ Anderson, Department of Pathology, University of Edinburgh Medical School, Teviot Place, Edinburgh EH8 9AG, Scotland.